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(57) Abstract

The present invention provides for a method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell. Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which includes: a) contacting the cell with the agent and amyloid-β peptide; b) determining the amount of amyloid-β peptide bound to the cell and c) comparing the amount of bound amyloid-β peptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

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A POLYPEPTIDE FROM LUNG EXTRACT WHICH BINDS AMYLOID-ß PEPTIDE

This application claims the priority of U.S. Serial No. 08/592,070, filed January 26, 1996, the contents of which are hereby incorporated by reference into the present application.

The invention disclosed herein was made with Government support under USPHS Grants No. AG00690, AG00603, HL21006 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding Sequence Listing and the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The pathologic hallmarks of Alzheimer's disease (AD) are intracellular and extracellular accumulations of proteins, progression of which is closely correlated with eventual neuronal dysfunction and clinical dementia (for reviews see Goedert, 1993; Haass et al., 1994; Kosik, 1994; Trojanowski et al., 1994; Wischik, 1989). Amyloid-ß peptide (Aß), which is the principal component of extracellular deposits in AD, both in senile/diffuse plaques and in cerebral vasculature, actively influences cellular functions as indicated by

several lines of evidence: Aß has been shown to promote neurite outgrowth, generate reactive oxygen intermediates (ROIs), induce cellular oxidant stress, lead to neuronal cytotoxicity, and promote microglial activation (Behl et al., 1994; Davis et al., 1992; Hensley, et al., 1994; Koh, et al., 1990; Koo et al., 1993; Loo et al., 1993; Meda et al., 1995; Pike et al., 1993; Yankner et al., 1990). For Aß to induce these multiple cellular effects, cell surfaces may contain a binding protein(s) which engages Aß. In this context, several cell-associated proteins, as well as sulfated proteoglycans, can interact with Aß. These include: substance P receptor, the serpin-enzyme complex (SEC) receptor, apolipoprotein E, apolipoprotein J (clusterin), transthyretin, alpha-1 anti-chymotrypsin, ß-amyloid precursor protein, and sulphonates/heparin sulfates (Abraham et al., 1988; Fraser et al., 1992; Fraser et al., 1993; Ghiso et al., 1993; Joslin et al., 1991; Kimura et al., 1993; Kisilevsky et al., 1995; Strittmatter et al., 1993a; Strittmatter et al., 1993b; Schwarzman et al., 1994; Snow et al., 1994; Yankner et al., 1990). Of these, the substance P receptor and SEC receptor might function as neuronal cell surface receptors for Aß, though direct evidence for this is lacking (Fraser et al., 1993; Joslin et al., 1991; Kimura et al., 1993; Yankner et al., 1990). In fact, the role of substance P receptors is controversial, and it is not known whether Aß alone interacts with the receptor, or if costimulators are also required (Calligaro et al., 1993; Kimura et al., 1993; Mitsuhashi et al., 1991) and the SEC receptor has yet to be fully characterized. Amyloid-ß peptide (Aß) is central to the pathology of Alzheimer's disease (AD), primarily because of its neurotoxic effects which involve induction of cellular oxidant stress.

Summary of the Invention

The present invention provides for a method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell. Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which includes: a) contacting the cell with the agent and amyloid-β peptide; b) determining the amount of amyloid-β peptide bound to the cell and c) comparing the amount of bound amyloid-β peptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

Brief Description of the Figures

Figures 1A, 1B, 1C, 1D, 1E and 1F. Colocalization of heme oxygenase type I (HO-1; A,D), p50 antigen (B, E) and RAGE (C,F) in proximity to deposits of Aß in AD brain. Figures 1A, 1B, and 1C demonstrate increased expression of HO-1, p50 and RAGE antigens, respectively, in temporal lobe. The red color shows the antigen under study (HO-1, p50 or RAGE) and the black color depicts localization of Aß. Arrows in Figure 1B depict nucelar localization of p50. Figures 1D-1F display the same antigens in cerebrovasculature from AD-derived brain. Marker bar: 50 μm (1A,1C,1D,1F); 30 μm (B); 125 μm (E). Figure 1G shows ELISA for RAGE antigen in homogenates of AD brain versus age-matched apparently normal control.

Figures 2A, 2B, 2C-1, 2C-2, 2D-1, 2D-2, 2E and 2F. Aß-induction of cellular oxidant stress and binding involves association with a cell surface, trypsin-sensitive polypeptide on endothelium (A,C,E) and cortical neurons (B,D,F). A,C,E. Endothelial cells were incubated with the indicated concentration of synthetic Aß (1-40) or ^{125}I-Aß, and induction of cellular oxidant stress was assessed by the TBARS assay (A) or radioligand binding studies were performed (C-1,E). Where indicated, cultures were pre-treated with probucol or N-acetylcysteine (NAC), or briefly exposed to trypsin (Figure 2C-2: +, treated with trypsin; -, controls not treated with trypsin). In Figure 2E, cross-linking of ^{125}I-Aß (100 nM) to the cell surface was performed with DSS. Lanes correspond to: (1) ^{125}I-Aß alone + endothelium (no DSS); (2) ^{125}I-Aß with excess unlabelled Aß (100-fold), DSS and endothelium; and (3) ^{125}I-Aß with DSS and endothelium; and, Figures 2B, 2D, 2F show the same experiments performed with primary cultures of rat cortical neurons instead of endothelium. Note that in Figure 2F, samples are: (1) as in E, above; (2) ^{125}I-Aß with DSS and

cortical neurons; and, (3) ^{125}I-Aß with excess unlabelled Aß, DSS and cortical neurons.

Figures 3A, 3B and 3C. Purification of cellular Aß-binding polypeptide(s). Tissue extract was subjected to chromatography on heparin (A) and hydroxylapatite (B) columns. OD_{280} of the fractions (solid line), the concentration of salt in the buffer (broken line), and fractions with peak ^{125}I-Aß binding activity (arrow/bar) are shown. In Figure 3C, fractions with peak ^{125}I-Aß binding activity from the hydroxylapatite column were subjected to SDS-PAGE (10%; nonreduced), and stained with Coomassie blue or sliced into 2 mm pieces, eluted, and assayed for specific ^{125}I-Aß binding activity.

Figures 4A, 4B, 4C, 4D, and 4E. Binding of ^{125}I-Aß (1-40) and related peptides to RAGE: dose-dependence (A-B), effect of RAGE blockade (C) and competition (D-E). A-B. Binding of ^{125}I-Aß to purified RAGE immobilized in microtiter wells was studied: 4A, the indicated concentration of RAGE was incubated for adsorption to wells (^{125}I-Aß, 100 nM); and 4B, the indicated concentration of ^{125}I-Aß was added to wells pre-incubated with RAGE (5 µg/well). Where indicated, wells with adsorbed RAGE were preincubated with trypsin as described in the text (Figure 4A). 4C, Binding of ^{125}I-Aß (100 nM) to RAGE adsorbed to microtiter wells was studied in the presence of the indicated concentration of sRAGE (2-, 20- or 50-fold molar excess), sVCAM-1 (20x), anti-RAGE IgG (10 or 50 µg/ml) or nonimmune IgG (10 µg/ml). Figure 4D-E. Competitive binding studies. Plates were coated with RAGE (5 µg/well) as above. In Figure 4D, wells were incubated with ^{125}I-Aß (100 nM) in the presence of one of the following unlabelled competitors (in each case, an 100-fold molar excess of unlabelled peptide to ^{125}I-Aß was added): Arg-Gly-Asp-Ser (Seq. I.D. No. 1)(RGDS), scrambled Aß (25-35), Aß (25-35), Aß (1-20), aggregated Aß (1-40), Aß

(1-40, fresh), and 0 denotes no addition. In Figure 4E, binding studies utilized ^{125}I-Aß (100 nM) and an 100-fold excess of unlabelled Aß-derived from AD brain vasculature or plaque core, or synthetic Aß (1-40). Data shown represent percent maximal specific binding, and the mean±SEM of triplicate determinations is shown. Maximal specific binding (100%) is that observed with ^{125}I-Aß (1-40; 100 nM) alone minus ^{125}I-Aß + 100-fold molar excess unlabelled Aß (1-40; freshly prepared). Data were analyzed by ANOVA Fisher's PLSD and the P values are * <0.01; ^ <0.02.

Figures 5A, 5B and 5C. Role of RAGE in binding of ^{125}I-Aß to mouse endothelial cells and cortical neurons: effect of sRAGE and anti-RAGE IgG. Figure 5A. Effect of sRAGE on binding of ^{125}I-Aß to endothelium and cortical neurons. Figure 5B-C. Effect of anti-RAGE IgG, at the indicated concentration, on binding of ^{125}I-Aß to endothelium (B) or cortical neurons (C). In each case, cell cultures were incubated with ^{125}I-Aß (100 nM) alone or in the presence of the indicated concentrations of sRAGE, anti-RAGE IgG or nonimmune IgG (NI; 20 µg/ml). The mean±SEM of quintuplicate determinations is shown. P values shown are # <0.01 and *<0.05

Figures 6A, 6B, 6C and 6D. Effect of RAGE blockade on Aß-induced cellular oxidant stress in endothelial cells (A,C) and PC12 cells (B,D). Figure 6A-B. Generation of TBARS. Endothelial (A) or PC12 (B) cells were incubated with Aß (1 µM) either alone or in the presence of the indicated amounts of anti-RAGE IgG, nonimmune IgG (NI; 20 µg/ml) or sRAGE (100 µg/ml). P values shown are *<0.01 and # <0.05. Figure 6C-6D. Activation of NF-kB. The same cells were incubated with Aß alone or with other additions (as above), nuclear extracts were prepared and studied by EMSA. For endothelial cells (C) and PC12 cells (D): Lane 1, ^{32}P-labelled NF-kB probe; 2, cells not exposed to Aß; 3,

cells exposed to Aß (500 nM); 4-5, cells exposed to Aß (500 nM; 4) in the presence of anti-RAGE IgG or nonimmune IgG (10 μg/ml in each case; lane 5); 6, extracts of cells exposed to Aß (500 nM) in the presence of 100-fold excess unlabelled NF-KB probe.

Figures 7A, 7B, 7C, 7D, 7E, 7F, 7G and 7H. Effect of RAGE blockade on Aß-induced cellular stress in PC12 cells. Cultures were exposed to the indicated concentrations of Aß (25-35) alone, or in the presence of either sRAGE, anti-RAGE IgG, or nonimmune (NI) IgG. Figure 7A-D. The effect of RAGE blockade on MTT reduction is shown: A, MTT reduction at different concentrations of Aß in the presence/absence of sRAGE (20-fold molar excess over Aß); B, MTT reduction at different concentrations of sRAGE (Aß , 1 μM); and, C-D, MTT reduction at different concentrations of Aß, anti-RAGE IgG or nonimmune IgG (10 μg/ml). P values shown are *<0.01 and #<0.05. In Figure 7D, the concentration of Aß is 1 μM. Figures 7E-H show micrographs depicting PC12 cells either incubated in medium alone (E) or medium containing the following: Aß (1 μM; F); Aß (1 μM) + sRAGE (50 μg/ml; G); or Aß (1 μM) + anti-RAGE IgG (20 μg/ml; H). Addition of nonimmune IgG in place of anti-RAGE IgG had no effect on Aß-induced morphologic changes in PC12 cells. Marker bar: 100 μm.

Figures 8A-8B. RAGE expression by microglial cells. A-B. Colocalization of CD68. Figure 8A shows mouse anti-human CD68 IgG (4 μg/ml) and RAGE. Figure 8B shows rabbit anti-human RAGE IgG (30 μg/ml) in microglia approximate to a senile plaque from AD brain (adjacent sections). Immunostaining was performed using the indicated primary antibodies.

Figures 9A-9B. BV-2 cells bind ^{125}I-Aß in a RAGE-dependent manner. Figure 9A. Dose dependence of binding. Figure 9B.

Inhibition of ^{125}I-Aß binding by blockade of RAGE using anti-RAGE F(ab')$_2$ or sRAGE, at the indicated concentrations, but not by nonimmune F(ab')$_2$. BV-2 cells (5x10^4/well) in minimal essential medium with bovine serum albumin (1%) were incubated with either ^{125}I-Aß alone (1.3x10^4 cpm/ng; prepared by the Iodogen method) or in the presence of one of the indicated additions at 4°C for 2 hrs. Wells were washed with Hanks balanced salt solution seven times, and cell-associated radioactivity was eluted with Nonidet P-40 (1%). Specific binding (total minus nonspecific) is shown and equilibrium binding data was analyzed as described using Enzfitter (Schmitdt, et al. 1992). Synthetic peptide was from Quality Controlled Biochemicals and AD-derived Aß was prepared (Roher, et al. 1993).

Figures 10A, 10B and 10C. Modulation of BV-2 migration by Aß: role of RAGE. Figure 10A. Soluble Aß (1-40 and 25-35) induces chemotaxis of BV-2 cells in dose-dependent manner: effect of anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ on Aß(1-40 and 25-35)-induced chemotaxis. Figure 10B. Haptotoxis: effect of Aß or albumin adsorbed to the upper surface of chemotaxis chamber membranes on migration of BV-2 cells to fMLP added to the lower chamber. Figure 10C. Anti-RAGE F(ab')$_2$ blocks haptotactic response to Aß(1-40) adsorbed to chemotaxis chamber membranes. BV-2 cells were preincubated with either anti-RAGE F(ab')$_2$ or nonimmune F(ab')$_2$ for 1 hr at 4°C, cells were washed with Hanks balanced salt solution, and then added to the upper compartment of chemotoxis chambers in which the upper surface of membranes were coated with Aß(1-40). BV-2 cells (5x10^3/well) were added to the upper compartment of microchemotaxis chambers for 4 hrs at 37°C, and then cells reaching the lower surface of the chemotaxis chamber membrane were visualized with Wright's stain and counted. Data are reported as cells per high-power field (HPF) based on counting nine fields/well and each experiment was performed in triplicated. Where

indicated, the upper surface of the membranes was coated with Aß(1-40) by floating in an Aß solution (50 nM) for 3 hrs at 37°C, followed by washing and drying prior to use (Schmidt, et al. 1993).

Figures 11A, 11B and 11C. Aß-induced TNFa production and NF-kB activation in BV-2 cells is prevented by blocking access to RAGE. Figure 11A. Aß-induction of TNFα transcripts by PCR. BV-2 cells (10^6) were incubated with Aß(25-35; 500 nM) for 4 hr at 37°C, and RNA was harvested. RT-PCR was performed using primers for murine TNFα (Clontech) and the housekeeping gene mouse ß-actin (Clontech) was stimultaneously amplified. Ethidium bromide-stained gels are shown. The positive control (the respective cDNA) and migration of standard is shown. Where indicated, BV-2 cells were pre-incubated with anti-RAGE $F(ab')_2$ or nonimmune (NI) $F(ab')_2$ (10 μg/ml in each case) for 1 hr at 37°C. Figure 11B. Aß(25-35; 1 μM) induction of TNFα antigen in BV-2 cells. Cultures were incubated for 18 hrs at 37°C with Aß, and cell-free supernatants were assayed for TNFα antigen (Biosource). Where indicated, BV-2 cells were exposed to anti-RAGE $F(ab')_2$ or nonimmune $F(ab')_2$ as above. Figure 11C. Aß(25-35)-induction of NF-kB activation in BV-2 cells. Lanes represent: (1) free probe (FP); (2) BV-2 cells with medium alone; (3) BV-2 cells + Aß; (4-5) BV-2 cells + Aß with anti-RAGE $F(ab')_2$ (2 μg/ml [4] and 10 μg/ml [5]); (6) BV-2 cells + Aß and nonimmune $F(ab')_2$ (10 μg/ml); and, (7) BV-2 cells + Aß studied in the presence of an 100-fold excess of unlabelled NF-kB probe. Cultures (10^7 cells) were exposed to Aß (1 μM) for 4 hrs at 37°C, nuclear extracts were prepared, and gel retardation assay was performed using a double-stranded DNA oligonucleotide for the consensus NF-kB site. Where indicated, BV-2 cells were pre-incubated with anti-RAGE $F(ab')_2$ or nonimmune $F(ab')_2$ (10 μg/ml) for 1 hr at 37°C and then washed twice prior to exposure to Aß.

Detailed Description of the Invention

The present invention provides for a method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell.

In the present invention the cell may be a neuronal cell, an endothelial cell, a glial cell, a microglial cell, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, an endothelial cell, a tumor cell, or a stem cell. The agent may be a peptide, a peptidomimetic, a nucleic acid or a small molecule. The peptide may be at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2). The peptide may be at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-(Seq. I.D. No. 3).

The agent may be a soluble receptor for advanced glycation end product. The agent may be a soluble extracellular portion of a receptor for advanced glycation end product, an antibody or portion thereof, wherein the antibody is capable of specifically binding to the receptor for advanced glycation endproduct. The antibody may be a monoclonal antibody or a polyclonal antibody. A portion of the antibody may be a Fab or a complementarity determining region or a variable region. The agent may be capable of specifically binding to the amyloid-β peptide. The agent

may bind to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

One embodiment of this invention is a method for inhibiting degeneration of a neuronal cell which includes contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the neuronal cell.

Another embodiment of this invention is a method for inhibiting formation of an amyloid-β peptide fibril on a cell which includes contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the cell.

Another embodiment of this invention is a method for inhibiting extracellular assembly of an amyloid-β peptide into a fibril which includes contacting the amyloid-β peptide with an agent capable of inhibiting interaction of an amyloid-β peptide with another amyloid-β peptide.

Another embodiment of this invention is a method for inhibiting aggregation of amyloid-β peptide on the surface of a cell which includes contacting the amyloid-β peptide with an agent capable of inhibiting interaction of the amyloid-β peptide with a receptor for advanced glycation end product.

Another embodiment of this invention is a method for inhibiting aggregation of amyloid-β peptide on the surface of a cell which includes contacting the receptor for advanced glycation end product with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product.

Another embodiment of this invention is a method for inhibiting infiltration of a microglial cell into senile plaques which includes contacting the microglial cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the microglial cell.

Another embodiment of this invention is a method for inhibiting activation of a microglial cell by an amyloid-β peptide which includes contacting the microglial cell with an agent capable of inhibiting interaction of the amyloid-β peptide with a receptor for advanced glycation end product on the surface of the microglial cell. The inhibition of activation may include decreased production of cytokines by the microglial cell. The interaction may be binding of the amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

One embodiment of this invention is a method for treating a subject with a condition associated with interaction of an amyloid-β peptide with a receptor for advanced glycation end product on a cell, which comprises administering to the subject an agent capable of inhibiting the interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit the amyloid-β peptide interaction with the receptor for advanced glycation end product on the cell thereby treating the subject. The condition in this embodiment may be diabetes, Alzheimer's Disease, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, multiple sclerosis or neuronal degeneration. The subject may be a mammal or a human. The administration in this embodiment may be intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical,

nasal, oral, anal, ocular or otic delivery.

In this embodiment, the condition may be associated with degeneration of a neuronal cell in the subject, with formation of an amyloid-β peptide fibril, with aggregation of amyloid-β peptide, with infiltration of a microglial cell into a senile plaque, or with activation of a microglial cell by an amyloid-β peptide, wherein the activation comprises production of cytokines by the microglial cell.

Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which includes: a) contacting the cell with the agent and amyloid-β peptide; b) determining the amount of amyloid-β peptide bound to the cell and c) comparing the amount of bound amyloid-β peptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

In this embodiment, the cell may be contacted with the agent and the amyloid-β peptide simultaneously or the cell may be contacted with the amyloid-β peptide and the agent. The agent may be capable of specifically binding to the amyloid-β peptide. The agent may bind to amyloid-β peptide at the site where the receptor for advanced glycation end product interacts. The agent may be a soluble extracellular portion of a receptor for advanced glycation end product. The agent may be bound to a solid support. The agent may be expressed on the surface of a cell.

Another embodiment of this invention is a method for inhibiting activation of an NF-κB gene in a cell which

comprises contacting the cell with an agent which is capable of inhibiting interaction of amyloid-β peptide with a receptor for advanced glycation endproduct on the cell, thus inhibiting activation of NF-κB in the cell.

The present invention provides for a pharmaceutical composition which comprises an agent capable of inhibiting interaction of amyloid-β peptide with a receptor for advanced glycation endproduct and a pharmaceutically acceptable carrier. The carrier may be a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

As used herein, the term "oxidant stress" encompasses the perturbation of the ability of a cell to ameliorate the toxic effects of oxidants. Oxidants may include hydrogen peroxide or oxygen radicals that are capable of reacting with bases in the cell including DNA. A cell under "oxidant stress" may undergo biochemical, metabolic, physiological and/or chemica modifications to counter the introduction of such oxidants. Such modifications may include lipid peroxidation, NF-kB activation, heme oxygenase type I induction and DNA mutagenesis. Also, antioxidants such as glutathione are capable of lowering the effects of oxidants.

As used herein, the term "neurotoxicity" encompasses the negative metabolic, biochemical and physiological effects on a neuronal cell which may result in a debilitation of the neuronal celluar functions. Such functions may include memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses), synapse formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. Neurotoxicity may include neuronal cytotoxicity.

As used herein, the term "neuronal degeneration" encompasses

a decline in normal functioning of a neuronal cell. Such a decline may include a decline in memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses), synapse formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. In the present invention, the subject may be a mammal or a human subject. The administration may be intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; gene bombardment; topical, nasal, oral, anal, ocular or otic delivery.

In the practice of any of the methods of the invention or preparation of any of the pharmaceutical compositions an "therapeutically effective amount" is an amount which is capable of inhibiting the binding of an amyloid-β peptide with a receptor for advanced glycation endproduct. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. For the purposes of this invention, the methods of administration are to include, but are not limited to, administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

This invention also provides for pharmaceutical compositions including therapeutically effective amounts of protein compositions and compounds capable of inhibiting the binding of an amyloid-β peptide with a receptor for advanced glycation endproduct in the subject of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in treatment of neuronal degradation due to aging, a learning disability, or a neurological disorder. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition. The choice of compositions will

depend on the physical and chemical properties of the compound capable of alleviating the symptoms of the cognitive disorder of memory or the learning disability in the subject.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Portions of the compound of the invention may be "labeled" by association with a detectable marker substance (e.g., radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detection and quantification of compound or its receptor bearing cells or its derivatives in solid tissue and fluid samples such as blood, cerebral spinal fluid or urine.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone

or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The compound of the present invention capable of alleviating symptoms of a cognitive disorder of memory or learning may be delivered in a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the

epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

The pathologic hallmarks of Alzheimer's disease (AD) are intracellular and extracellular deposition of filamentous proteins which closely correlates with eventual neuronal dysfunction and clinical dementia (for reviews see Goedert, 1993; Haass et al., 1994; Kosik, 1994; Trojanowski et al., 1994; Wischik, 1989). Amyloid-ß peptide (Aß) is the principal component of extracellular deposits in AD, both in senile/diffuse plaques and in cerebral vasculature. Aß has been shown to promote neurite outgrowth, generate reactive oxygen intermediates (ROIs), induce cellular oxidant stress, lead to neuronal cytotoxicity, and promote microglial activation (Behl et al., 1994; Davis et al., 1992; Hensley, et al., 1994; Koh, et al., 1990; Koo et al., 1993; Loo et al., 1993; Meda et al., 1995; Pike et al., 1993; Yankner et al., 1990). For Aß to induce these multiple cellular effects, it is likely that plasma membranes present a

binding protein(s) which engages Aß. In this context, several cell-associated proteins, as well as sulfated proteoglycans, can interact with Aß. These include: substance P receptor, the serpin-enzyme complex (SEC) receptor, apolipoprotein E, apolipoprotein J (clusterin), transthyretin, alpha-1 anti-chymotrypsin, ß-amyloid precursor protein, and sulphonates/heparan sulfates (Abraham et al., 1988; Fraser et al., 1992; Fraser et al., 1993; Ghiso et al., 1993; Joslin et al., 1991; Kimura et al., 1993; Kisilevsky et al., 1995; Strittmatter et al., 1993a; Strittmatter et al., 1993b; Schwarzman et al., 1994; Snow et al., 1994; Yankner et al., 1990). Of these, the substance P receptor and SEC receptor might function as neuronal cell surface receptors for Aß, though direct evidence for this is lacking (Fraser et al., 1993; Joslin et al., 1991; Kimura et al., 1993; Yankner et al., 1990). In fact, the role of substance P receptors is controversial, and it is not known whether Aß alone interacts with the receptor, or if costimulators are required (Calligaro et al., 1993; Kimura et al., 1993; Mitsuhashi et al., 1991) and the SEC receptor has yet to be fully characterized.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Example 1: A Receptor Mediating Amyloid-ß Peptide-induced Oxidant Stress and Neurotoxicity

Amyloid-ß peptide (Aß) is central to the pathology of Alzheimer's disease (AD), primarily because of its neurotoxic effects which involve induction of cellular oxidant stress. A cell-associated ≈50 kDa polypeptide which binds Aß peptide (1-40) was purified to homogeneity and was shown to be identical to RAGE, an immunoglobulin superfamily member which also serves as a receptor for both amphoterin, a group I DNA binding protein involved in neuritic outgrowth, and advanced glycation endproducts (AGEs). Recombinant RAGE bound ^{125}I-Aß (1-40) in a dose-dependent manner; binding was blocked by excess Aß purified from AD brain and by unlabelled synthetic Aß (1-40 and 25-35) but not by Aß (1-20). Binding of ^{125}I-Aß to cultured cortical neurons was prevented by anti-RAGE IgG or excess soluble RAGE. In addition, blocking access to RAGE prevented Aß-induced cellular oxidant stress and cytotoxicity. Increased expression of RAGE in neurons proximate to senile plaques in AD brain, which also display evidence of oxidant stress and cytotoxicity, further suggests the relevance of RAGE-Aß interaction to cellular dysfunction and ultimate neurodegeneration, and is consistent with the concept that this interaction might be critical in the pathogenesis of AD.

Introduction

The possibility exists that another/other cellular binding site(s) might be present which could tether Aß to the cell surface. The importance of identifying such receptors is evident from the probability that Aß closely associated with the cell surface might trigger reactions generating potent

oxidizing stimuli capable of inducing cytotoxicity. If this hypothesis is correct, blocking access of Aß to the cell surface binding site(s) might considerably retard progression of cellular dysfunction in AD.

This study reports the isolation and characterization of a ~50 kDa polypeptide which binds synthetic Aß peptide (1-40) and is identical to RAGE (the Receptor for Advanced Glycation Endproducts). RAGE, previously identified as a central cellular receptor for Advanced Glycation Endproducts (AGEs; Schmidt et al. 1992; Neeper et al., 1992) also serves as a neuronal receptor for amphoterin, a molecule involved in neurite outgrowth (Rauvala et al., 1987). It is shown herein that RAGE mediates the interaction of Aß with endothelial cells (ECs) and neuronal-like cells, as demonstrated by radioligand binding experiments, as well as by its ability to mediate cellular oxidant stress and neuronal cytotoxicity. Enhanced expression of RAGE in AD, both in affected neurons and the vasculature, is consistent with the possibility that Aß-RAGE interaction may be relevant to the cytotoxicity central to this dementia.

Results

Relationship between oxidant stress markers and Aß in AD brain. Previous studies have demonstrated an association between neuronal injury in AD and oxidant stress (Coyle et al., 1993; Hensley, 1994; Volicer et al., 1990) by expression of heme oxygenase type 1 (HO-1), nuclear localization of the p50 subunit of NF-kB, and expression of malondialdehyde-lysine epitopes (Smith et al., 1994; Yan et al., 1994; Yan et al., 1995). Although extracellular Aß can induce oxidant stress in neurons *in vitro* (Behl et al., 1994; Hensley et al., 1994), this would probably be attenuated *in vivo* by the presence of abundant antioxidants. Nevertheless, neurons proximate to senile plaques displayed HO-1, and elevated levels of the p50 subunit of the NF-kB

family (Fig. 1A-B, respectively). Increased expression of p50 was especially prominent in a nuclear distribution (see arrows in Fig 1B), suggestive of its activation. Deposition of Aß also occurs in the cerebral vasculature (Glenner, 1979; Kawai et al., 1992; Luthert et al., 1991; Yamaguchi et al., 1992); oxidant stress (increased HO-1 and p50) was also evident in those vessels with accumulated Aß (Fig. 1D-E). Expression of HO-1 and p50 was enhanced in both endothelial cells and smooth muscle cells. In contrast, cerebral vasculature from age-matched controls showed minimal staining for HO-1 and p50. These observations suggested a spatial association between Aß deposition and the expression of markers of enhanced cellular oxidant stress in AD brain, leading to the hypothesis that binding of Aß to specific cellular structures might promote such perturbation of cellular properties, in spite of the antioxidant milieu prevalent *in vivo*.

Characterization of the interaction of Aß with ECs and neuronal-like cells. Signs of oxidant stress in AD lead to the interaction of Aß with vascular cells and with neurons, and the consequences for perturbation of cellular functions. Incubation of synthetic Aß (1-40) with ECs from murine cerebral vasculature or rat cortical neurons cells resulted in a dose-dependent increase in thiobarbituric acid-reactive substances (TBARS), which was blocked by pre-treatment of cultures with probucol or N-acetylcysteine (NAC) suggestive of an oxidant-sensitive mechanism (Fig. 2A-B). These results are consistent with what has been reported previously with neurons exposed to Aß (Behl et al., 1994; Harris et al., 1995; Mattson et al., 1995). To test the possibility that Aß-induced cellular oxidant stress involved interaction with a cell surface polypeptide, endothelial cells or cortical neurons were briefly treated with trypsin, the enzyme was neutralized and cells then exposed to Aß. Trypsin treatment virtually completely blocked appearance of

TBARS (Fig. 2A-B), though it had no effect on detection of TBARS when added after cells had been pre-incubated with Aß. Similar experiments examining induction of HO-1 mRNA and nuclear translocation of NF-kB showed cellular activation by Aß, which was prevented by pretreatment of cultures with trypsin. Studies with PC12 cells provided similar results. These data strongly supported a role for a trypsin-sensitive cell surface polypeptide in Aß-induction of cellular oxidant stress.

Reasoning that such a cell surface structure was likely to function, at least in part, as a binding site to tether Aß to the cell, radioligand binding studies were performed. When synthetic Aß peptide (residues 1-40) was radiolabelled, specific and dose-dependent binding of ^{125}I-Aß to cultured ECs was observed (Fig. 2C). Half-maximal binding occurred at an ^{125}I-Aß concentration of 40.0±9.79 nM and, at saturation, there were ≈1.7x10^{6} molecules bound per cell. Exposure of cultures to trypsin blocked binding of ^{125}I-Aß (Fig. 2C, inset). Concentrations of ^{125}I-Aß which increased occupancy of cell surface binding sites were comparable to those inducing endothelial generation of TBARS (Fig. 2A). Further supporting a central role for a cell surface binding site is clear from the strong inhibitory effect of trypsin on both ^{125}I-Aß binding and Aß-mediated oxidant stress. Similarly, radioligand binding studies on cultured rat cortical neurons displayed dose-dependent ^{125}I-Aß binding, K_d 55.2±14.6 nM, which was blocked by pre-treatment of cells with trypsin (Fig. 2D).

To further identify the nature of cell surface polypeptide(s) with which ^{125}I-Aß was interacting, crosslinking studies were performed. ECs with surface-bound ^{125}I-Aß were exposed to disuccinimidyl suberate (DSS; 0.2 mM) and extracts were prepared for SDS-PAGE and autoradiography (Fig. 2E). A single major band, M_r ≈50-55 kDa was observed

(Fig. 2E, lane 3), whose intensity was greatly attenuated upon addition of an 100-fold molar excess of unlabelled Aß (Fig. 2E, lane 2). Ruling out the possibility that this band was due to self-aggregation of the tracer, when ^{125}I-Aß was incubated with DSS under the same conditions in the absence of ECs, no band was observed. Similar cross-linking studies on cortical neurons also showed that ^{125}I-Aß became associated with a polypeptide of M_r ≈50-55 kDa (Fig. 2F, lane 2).

Before proceeding further with characterization of the ≈55 kDa polypeptide, it was important to determine whether cell-associated ^{125}I-Aß was interacting with previously recognized Aß-binding proteins, such as apolipoproteins E and J, heparin-like and heparan sulfate proteoglycans, substance P receptors, and transthyretin. Pre-treatment of ECs with heparin (10 µg/ml), which has been shown to release cell-associated apo E (Ji et al., 1994) or preincubation with heparanase (under conditions which blocked 80% of ^{125}I-basic fibroblast growth factor binding to ECs, due to cell surface and matrix heparan sulfate proteoglycans; Moscatelli, 1992) did not affect ^{125}I-Aß binding to endothelium. Pilot studies in which substance P (added as an unlabelled competitor) or antibody to transthyretin was present during the incubation of ^{125}I-Aß with ECs had no effect on binding. Apo J, with M_r ≈80 kDa (Ghiso et al., 1993), was unlikely to be responsible for the ≈55 kDa band observed on SDS-PAGE following crosslinking of ^{125}I-Aß to the cell surface. These studies suggested that the cell-associated Aß binding polypeptide under study here was distinct from previously described species.

Isolation and characterization of Aß cell-associated binding proteins. In order to purify cellular Aß binding proteins, a binding assay was developed employing extracts of cultured cells or tissues immobilized on microtiter wells and ^{125}I-Aß.

The first studies used ECs, as their binding of ^{125}I-Aß was similar to that of neurons, and because they are easier to culture and obtain in larger numbers. ECs were extracted with octyl-ß-glucoside in the presence of protease inhibitors, diluted in carbonate/bicarbonate buffer, and the mixture was adsorbed to microtiter wells. Specific binding of ^{125}I-Aß was similar to that observed on cultured ECs: it was dose-dependent (K_d 32.9±10.2 nM) and completely prevented by pre-treatment of the cell extract with trypsin. To further scale up starting material for purification of cell-associated Aß binding proteins, acetone-extract of bovine lung was tested using a similar assay following adsorption to microtiter wells and incubation with ^{125}I-Aß. Again, similar specific, trypsin-sensitive, and dose-dependent binding was observed. Although extract of brain, especially from patients with AD, would have been a more appropriate starting material for purification of Aß binding polypeptides, studies showed that brain extract, though in many respects comparable to lung extract, was not a convenient starting material. Results of binding assays with brain extract were not reproducible, probably due to the high content of lipid, and human brain, especially patient-derived tissue, was not available in sufficient quantity.

The preparation of Aß binding protein(s) from lung was performed by subjecting acetone extract of the tissue to sequential chromatography on heparin and hydroxylapatite columns. Following application of tissue extract, the heparin column was washed with equilibration buffer and eluted with ascending concentrations of NaCl (Fig. 3A). ^{125}I-Aß binding activity was observed in the eluate, as indicated (maximal activity, 0.5 M NaCl), and fractions were pooled, dialyzed and applied to hydroxylapatite. The column was eluted with increasing concentrations of phosphate, and fractions corresponding to 0.25 M displayed the highest

binding activity for ^{125}I-Aß (Fig. 3B). This material was concentrated and applied to preparative nonreduced SDS-PAGE (10%), and two major bands were observed corresponding to M_r's of 30-35 and 50-60 kDa (Fig. 3C). Another lane of the same gel loaded with the same sample was sliced into 2 mm pieces, and each piece subjected to gel elution; the eluate was studied in the ^{125}I-Aß binding assay. Each of the two major protein bands demonstrated the capacity to specifically bind ^{125}I-Aß (Fig. 3C). In contrast, gel elution of lanes in which molecular weight standards were run in place of test samples revealed no binding of ^{125}I-Aß.

Using the same gel system as above, bands were cut-out and subjected to N-terminal sequence analysis. The ≈50 kDa band was sequenced first as it was most abundant. The sequence revealed virtual identity with bovine RAGE (Table 1).

Table 1 - Amino acid sequences of the ≈50KdA GEL BAND AND 30-35 kDa gel band: Comparison to RAGE

RAGE NH_2-terminal	DQNITARIGKPLVLNCKGAPKKPPQQLEWK[a]
NH_2-terminal ≈50kDa gel	DQXITARIGKPLVLNXKGAPKKPPQQLEW(K)[b]
NH_2-terminal 30-35 kDa gel band	DQXITARIGKPLVLNXKGAP[c]

The following abbreviations were used: X, no residue identifiable at that cycle; parenthesis, tentative assignment. (Neeper et al. 1992). [a]Seq. I.D. No. 8; [b] Seq. I.D. No. 9; [c]Seq. I. D. No. 10)

Based on protein structure deduced from the bovine RAGE cDNA and immunoblotting results (Neeper et al., 1992; Brett et al., 1993), the ≈50 kDa band would most likely correspond to full-length RAGE. The 30-35 kDa band also demonstrated the same N-terminal sequence as RAGE, and most likely represented the extracellular domain of RAGE which has been observed previously to undergo proteolysis during purification (Schmidt et al., 1992). Consistent with these data, both 30-35 and ≈50 kDa polypeptides were reactive with

anti-RAGE IgG prepared against recombinant human RAGE. A minor, more slowly migrating peak of Aß binding activity, corresponding to M_r ≈66 kDa (Fig. 3C), gave two N-terminal sequences, that for RAGE and albumin.

Binding of ^{125}I-Aß to RAGE. The interaction of RAGE with Aß was further characterized by radioligand binding studies, using purified recombinant human RAGE (a form consisting of the extracellular domain termed soluble or sRAGE) expressed in a baculovirus system (Hori et al., 1995). Following adsorption of RAGE to microtiter wells, specific binding of ^{125}I-Aß (binding observed with ^{125}I-Aß alone minus that observed with ^{125}I-Aß and excess unlabelled Aß) was dependent on the concentration of RAGE incubated with the wells (Fig. 4A). No specific binding was observed when RAGE was replaced by albumin. Exposure of RAGE-coated wells to trypsin (Fig. 4A) completely blocked subsequent binding of ^{125}I-Aß, as expected from our previous observation that RAGE is inactivated by trypsin (Schmidt et al., 1992). When the concentration of RAGE adsorbed to wells was held constant, and varying amounts of ^{125}I-Aß were added, binding was dose-dependent with half-maximal occupancy of binding sites at 56.8±13.9 nM (Fig. 4B). This was similar to the range of Aß concentrations resulting in binding to and oxidant stress in endothelial cells, PC12 cells and cortical neurons. Addition of either anti-RAGE IgG or soluble RAGE simultaneous with ^{125}I-Aß resulted in dose-dependent inhibition of binding (Fig. 4C), whereas nonimmune (NI) rabbit IgG or another immunoglobulin superfamily molecule, vascular cell adhesion molecule-1 (VCAM-1; Osborn, 1989) was without effect. These data indicate that RAGE binds synthetic ^{125}I-Aß peptide (1-40).

Competition studies were performed to assess the region of Aß (1-40) interacting with RAGE and to evaluate the effect of Aß purified from AD brain (Fig. 4D-E). In the presence

of freshly-prepared unlabelled Aß (1-40), binding of ^{125}I-Aß to RAGE was blocked (Fig. 4D). Competition was also observed if Aß (1-40) was allowed to incubate for 3 days at 37°C, conditions which promote aggregation of the peptide (Hensley et al., 1994; Lorenzo et al., 1994). A truncated form of Aß (1-40) consisting of residues 25-35 also inhibited binding of ^{125}I-Aß to RAGE. In accord with the specificity of Aß-RAGE interaction, addition of excess unlabelled Aß (1-20), scrambled Aß (25-35), or an irrelevant peptide such as Arg-Gly-Asp-Ser (Seq. I.D. No. 1) had no effect on ^{125}I-Aß binding to RAGE (Fig. 4D). Furthermore, human Aß (1-40/1-42), chromatographically-purified from neuritic plaques or vascular amyloid from brains of patients with AD (Roher et al., 1993a; Roher et al., 1993b), inhibited the binding of ^{125}I-Aß (Fig. 4E).

As a first step in understanding whether RAGE would mediate the binding of Aß to cells, radioligand binding studies were performed with cultured mouse brain endothelial cells (Fig. 5A,5B) and rat cortical neurons (Fig. 5A,5C). In each case, binding of ^{125}I-Aß was blocked by addition of excess soluble RAGE or anti-RAGE IgG in a dose-dependent manner. These data indicated that RAGE functioned as a binding site for ^{125}I-Aß on cellular surfaces.

RAGE and Aß-induced cellular stress. A series of experiments suggested that Aß interaction with RAGE mediates cellular perturbations resulting in oxidant stress and cytotoxicity. Endothelial cells exposed to Aß generated TBARS (Fig. 2A and 6A); this was prevented by blocking access to RAGE using either anti-RAGE IgG or excess soluble receptor (Fig. 6A). Another index of oxidant stress triggered by Aß, activation of NF-kB in ECs, was also suppressed by blocking RAGE (Fig. 6C, compare lanes 3 and 4). Similar experiments on PC12 cells with Aß showed generation of TBARS (Fig. 6B) and activation of NF-kB (Fig.

6D, compare lanes 3 and 4), also prevented by blocking interaction of Aß with the receptor.

Because Aß is well-known for its toxicity to a range of cultured neurons and neuronal-related cells (Behl et al., 1994; Harris et al., 1995; Hensley et al., 1994; Koh et al., 1990; Loo et al., 1993; Lorenzo et al., 1994; Pike et al., 1993; Yankner et al., 1990), it was important to determine if Aß-RAGE interaction mediated such effects of the peptide. Incubation of PC12 cells with Aß (25-35) resulted in dose-dependent cytotoxic/stress-associated changes, including perturbation of cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT; Fig. 7A). This biochemical index of cellular stress was accompanied by morphologic changes, including retraction of cellular processes (Fig. 7, compare panels E-F). Addition of sRAGE (Fig. 7A-B, G) or anti-RAGE IgG (Fig. 7C-D, H) prevented Aß-induced cellular stress, based on each of these criteria. Similar results were observed when PC12 cells were incubated with Aß(1-40).

RAGE expression in AD. The above data suggested that RAGE, if present and/or up-regulated in cells likely to be critical in the pathogenesis of AD, might participate in mediating toxic effects when associated with Aß and related peptides. ELISA of homogenates of AD brain demonstrated ~2.5-fold increase in patients compared with age-matched controls (Fig. 1G). Immunocytochemical studies showed increased expression of RAGE (Fig. 1C) in neurons displaying evidence of oxidant stress (Fig. 1A-B) proximate to deposits of Aß. In the vasculature, RAGE expression was also enhanced in the vessel wall (Fig. 1F), in cellular elements showing increased HO-1 and p50 (Fig. 1D-E) in the vicinity of Aß deposition. Closer examination of vessel walls showed RAGE to be enhanced in both endothelium and smooth muscle cells. In contrast, RAGE expression was minimal in

age-matched cerebral vasculature from apparently normal controls. Thus, RAGE expression appears to be significantly upregulated in neuronal elements around or participating in the neuritic plaques as well as in the vascular cells of the affected AD brain.

Discussion

RAGE is a member of the immunoglobulin superfamily of cell surface molecules originally identified by its capacity to bind advanced glycation endproducts (AGEs; Schmidt et al., 1992; Neeper et al., 1993). These irreversibly nonenzymatically glycated adducts, which form on free amino groups, accumulate in tissues during normal aging, and their deposition is accelerated in diabetes, renal failure, and settings in which macromolecular turnover is delayed (Baynes, 1991; Ruderman et al., 1992). As AGEs have been localized to the vasculature, especially in diabetes and experimental hyperlipidemic atherosclerosis (Nakamura et al., 1993; Palinski et al., 1995), the first characterization of RAGE in these studies emphasized its role as a cell surface receptor for AGEs on endothelial cells and mononuclear phagocytes. AGE-RAGE interaction mediates perturbation of central properties of each of these cell types for vascular homeostasis: vascular permeability is increased, endothelial expression of VCAM-1 is induced, monocyte migration is stimulated and production of proinflammatory cytokines, such as tumor necrosis factor-alpha occurs (Schmidt et al., 1993; Schmidt et al., 1995; Wautier et al., 1995;).

In addition to the vasculature, expression of RAGE is especially prominent in the central nervous system (Brett et al., 1993; Hori et al., 1995). During the embryonic and early postnatal period in rat brain, RAGE is highly expressed by cortical neurons in hippocampus (P5) and

cerebellum (P17). These observations strongly suggested that AGEs were almost certainly not the primary ligands of RAGE, and led to studies to seek another ligand which could engage RAGE and affect cellular function, distinct from those consequent on AGE-RAGE interaction. This ligand proved to be amphoterin, a high mobility group I DNA binding protein which is highly expressed in developing neurons and has been shown to mediate neurite outgrowth when present extracellularly (Rauvala et al., 1987). Amphoterin binds to RAGE specifically and with high affinity (K_d ~9 nM; Hori et al., 1995); radioligand binding studies with ^{125}I-amphoterin showed RAGE to be the receptor mediating its binding to cortical neurons, as well as subsequent induction of neurite outgrowth. Furthermore, neurons containing RAGE in developing rat cerebral cortex also expressed, or were contiguous to other cells which expressed amphoterin, suggesting that amphoterin-RAGE interaction was occurring under physiologic conditions (Hori et al., 1995). Although detailed neuroanatomical studies of RAGE expression have not yet been done, the receptor clearly displays more limited expression in the adult central nervous system compared with the early perinatal period. In adult animals, RAGE is found in a population of cortical neurons yet to be characterized and in spinal motor neurons (Brett et al., 1993). In Alzheimer's disease, expression of RAGE within cortical neurons increases and is more widespread, especially in those neurons proximate to deposits of Aß and those cells bearing neurofibrillary tangles. This suggested that there was yet another ligand for RAGE under pathologic conditions, such as in AD brain.

As findings concerning RAGE expression in AD were emerging, independent investigation into how Aß induced cellular oxidant stress was ongoing. Aß itself has been shown to generate ROIs (Hensley et al., 1994) and Aß interaction with PC12 cells additionally triggers intracellular formation of

oxidants (Behl et al., 1994), such as hydrogen peroxide, resulting in perturbation of a range of cellular properties. Expression of markers associated with cellular oxidant stress in AD brain has been demonstrated, including HO-1, activation of NF-kB and the presence of malondialdehyde-lysine epitopes (Smith et al., 1994; Yan et al., 1994; Yan et al., 1995). As antioxidant mechanisms abound in the extracellular space, it seemed that Aß engagement of a cell surface polypeptide would tether Aß to the membrane where it could exert its oxidant effects in proximity to cellular structures. Consistent with this concept, binding of Aß to cortical neurons parallelled the induction of cellular oxidant stress, and both were blocked by pre-treatment of cultures with trypsin. Two lines of investigation, one to understand the role of RAGE in AD brain and the other to identify cellular binding site(s) for Aß that mediate induction of cellular oxidant stress, converged when the N-terminal sequence of the cellular Aß binding protein proved to be identical to RAGE. Experiments with recombinant RAGE and blocking antibody to the receptor further confirmed that RAGE specifically bound Aß and mediated, in large part, Aß binding to cortical neurons, as well as Aß induction of oxidant stress and neurotoxicity.

The interaction of Aß with RAGE was dose-dependent and specific, and appeared to involve the region comprising residues 25-35 of the peptide. As this portion of Aß has been shown to participate in Aß-mediated cytotoxicity and self-aggregation (Loo et al., 1993; Hensley et al., 1994; Mattson et al., 1995; Shearman et al., 1994), it is possible that RAGE might act as a magnet to localize forming amyloid fibrils to the cell surface. Such a role for RAGE would emphasize its capacity for promoting the cytotoxicity of Aß, but may reflect an initially protective mechanism, intended to mediate clearance of Aß monomers or small aggregates, gone awry in a sea of large Aß aggregates in AD brain.

Addition of excess extracellular domain of RAGE (sRAGE) completely blocked ^{125}I-Aß binding to both ECs and cortical neurons. When present in sufficient molar excess to Aß, sRAGE also completely prevented Aß-induced neuronal toxicity; at higher levels of Aß, e.g. 10 μM, sufficient concentrations of soluble receptor could not be achieved for complete neuroprotection. Anti-RAGE IgG prevented ^{125}I-Aß binding to cortical neurons by only ≈70%, whereas under the same conditions it completely blocked ^{125}I-Aß binding to ECs. Several cell surface polypeptides/proteoglycans have been previously postulated to interact with Aß, including SEC, substance P receptors and heparan sulfate proteoglycans, although their role in the pathobiology of AD is not well established. The inability to completely block specific binding of ^{125}I-Aß to cortical neurons suggests that these or other binding sites may also be involved, in addition to RAGE. It is important to note that sRAGE, once bound to Aß, appears to block cellular interactions of amyloid-ß peptide, whether the latter is present as monomer or under conditions favoring aggregation. This could reflect the size of sRAGE, an μ30 kDa polypeptide compared with Aß (-4 kDa), but it also might be due to binding in the vicinity of Aß (25-35). It is possible that sRAGE can diminish cellular interactions and toxicity of Aß deposits by preempting neuronal interactions (with cell surface RAGE and, potentially, other cell surface binding sites) and possibly by limiting assembly of Aß into aggregates/fibrils. As blocking fibril formation may delay pathologic changes in AD, and as sRAGE-Aß monomers or smaller aggregates might turn over more rapidly, a detoxification mechanism is suggested.

Aß is an oxidizing agent, as are AGEs, and such ligands could exert their cellular effects simply as the result of being tethered to RAGE on the cell surface. However, recent studies have shown that stimulation of this receptor by other ligands which do not themselves generate ROIs, induces

intracellular generation of oxidants in target cells, suggesting that recruitment of signal transduction mechanisms may occur following ligand-RAGE interaction. Further support for an intimate relationship between RAGE and ROIs is the presence of three NF-kB sites in the RAGE promoter whose activity can be triggered by oxidants. These data suggest a possible feedback loop whereby binding of Aß to RAGE induces oxidant stress which further up-regulates RAGE, thereby augmenting further Aß-RAGE interaction. The presence of nonenzymatically glycated aggregates of Aß, which has also been shown in AD (Vitek et al., 1994), might additionally facilitate the interaction of extracellular Aß with RAGE.

Although these studies represent only a first step in establishing a link between expression of RAGE and pathophysiologic changes in AD, they suggest a novel paradigm in which engagement of RAGE has quite different outcomes depending on the nature of the ligand and the microenvironment. During the prenatal and early postnatal period, amphoterin-RAGE interaction promotes neurite outgrowth and supports normal development. In this context, small quantities of soluble Aß, which exists in normal individuals, might promote neurite outgrowth in association with RAGE. In the vicinity of neuritic plaques, where dystrophic neurites are present, Aß-RAGE could mediate a repair mechanism or stimulate de novo neurite outgrowth. However, engagement of RAGE on cortical neurons in the presence of higher levels of Aß in AD, and, by analogy, RAGE binding of AGEs in diabetic vasculature, could result in sustained cellular oxidant stress with deleterious consequences for cellular function and organic homeostasis.

Experimental Procedures

Cell culture, binding assays, and cross-linking studies.

Murine microvascular endothelial cells (ECs) were used in this studies (Gumkowski et al., 1987). Cultured rat cortical neurons, obtained from neonatal animals and cultured as described previously (Yavin et al., 1974) were used by day 17. At this time, cultures consisted of >90% neurons based on neurofilament staining. PC12 cells were obtained from ATCC (Rockville MD).

Aß (1-40), obtained from Quality Controlled Biochemicals (Hopkinton MA), was dissolved in distilled water (1 mg/ml) and radioiodinated by the Iodobead procedure (Pierce, Rockford IL) as described by the manufacturer. The reaction mixture was desalted by gel filtration, and ^{125}I-Aß was >90% precipitable in trichloroacetic acid (20%) and migrated as a single band with M_r ≈4 kDa on tris/tricine gel (16%) electrophoresis. Radioligand binding studies were performed immediately after labelling using endothelial cells and cortical neurons as above. Because of detachment of cortical neurons from the growth substrate during pilot binding experiments, cultures were briefly exposed to paraformaldehyde (2% for 15 min) prior to the binding studies. Cultures were washed with phosphate-buffered saline (calcium-magnesium-free), followed by addition of binding buffer (minimal essential medium containing bovine serum albumin-fatty acid free [1%]). Tracer, ^{125}I-Aß, was added either alone, in the presence of an 100-fold molar excess of unlabelled Aß or with one of the reagents listed below, and cultures were incubated (37°C for 2 hrs for neurons and 4°C for 3.5 hrs for ECs) to allow binding. This incubation time was sufficient for binding to reach an apparent maximum at the lowest tracer concentrations. Unbound ligand was removed by four rapid washes (a total of 6 secs/well); during washing, only a negligible amount of cell-bound ligand dissociated. Elution buffer (NaCl, 0.1 M, containing Nonidet P-40, 1%) was then added to wells for 5 min at 37°C, and the liquid aspirated and counted. Specific

binding was defined as total binding (counts observed in wells incubated with ^{125}I-Aß alone) minus nonspecific binding (counts observed in wells incubated with ^{125}I-Aß in the presence of an 100-fold excess of unlabelled Aß). Equilibrium binding data were analyzed according to the equation of Klotz and Hunston (Klotz et al., 1971) using nonlinear least-squares analysis. Where indicated, one of the following was added to binding mixtures: substance P (Sigma), rabbit anti-bovine RAGE IgG (Schmidt et al., 1992), nonimmune rabbit and human IgG (Sigma), recombinant soluble human RAGE, recombinant soluble murine vascular cell adhesion molecule-1 (VCAM-1), antibody to transthyretin (Sigma), and unlabelled Aß-related peptides (1-40 and 25-35). Note that anti-bovine RAGE IgG has been previously shown to interact with rat, human and bovine RAGE (Brett et al., 1993; Hori et al., 1995). In certain experiments, cells were pretreated with heparin (10 µg/ml for 30 min), heparanase (2.5 U/ml for 30 min; Sigma; Moscatelli, 1992), or TPCK-treated trypsin (0.05 units/ml for 30 min and serum was added to neutralize trypsin prior to the binding assay with ^{125}I-Aß; Sigma). None of these procedures resulted in cell detachment or loss of viability, based on trypan blue uptake or detachment from the growth substrate. Cross-linking experiments were performed on ECs and neurons (for these studies neurons were not exposed to paraformaldehyde) following binding of ^{125}I-Aß to the cell surface by adding disuccinimidyl suberate (DSS; 0.2 mM; Pierce) for 30 min at 25°C. Cultures were then washed twice and dissolved in lysis buffer (NaCl, 0.1 M; NP-40, 1%, PMSF, 2 mM, and leupeptin, 10 µg/ml) at 4°C for 1 hr. Cellular debris was removed by centrifugation (10,000 rpm for 10 min) and the supernatant was prepared for nonreduced SDS-PAGE (10%).

Endothelial cell protein extracts were prepared as described (Schmidt et al., 1992) from ≈10^8 cells using

octyl-ß-glucoside in the presence of protease inhibitors. Binding of ^{125}I-Aß to proteins in the extract was studied by a previously described method (Schmidt et al., 1992) using an incubation time of 3 hrs at 37°C. Other additions were as indicated above; note that Aß (1-20), scrambled Aß peptide (25-35) was from Quality Controlled Biochemicals, Arg-Gly-Asp-Ser (Seq. I.D. No. 1) was from Sigma, and Aß purified from AD brain plaque and vasculature was prepared as described (Roher et al., 1993a,b). For certain experiments, after adsorption of sRAGE to the wells and blocking with albumin, trypsin was added (0.5 units/ml for 1 hr; the initial amount of sRAGE added was 120 µg/ml), and neutralization of residual enzyme was as described above.

Purification of Aß binding proteins and characterization of Aß interaction with RAGE. Acetone extracts of bovine lung and brain (Sigma) were diluted in carbonate/bicarbonate buffer, adsorbed to microtiter wells (as above for endothelial cell extract), and adsorbed proteins studied for their ability to bind ^{125}I-Aß by the procedure described above. For purification of Aß binding proteins, lung extract (100 g) was dissolved in tris-buffered saline containing octyl-ß-glucoside (1%) and PMSF (2 mM) overnight at 4°C with constant agitation. The mixture was centrifuged (10,000 rpm for 30 min), applied to Heparin HyperD® (bed volume 40 ml; Biosepra), and the column eluted with increasing concentrations of NaCl. Fractions were studied in the microtiter assay for ^{125}I-Aß binding activity and those with maximal activity were pooled, dialyzed, and then loaded on hydroxylapatite-ultragel (30 ml bed volume; Biosepra). The column was eluted with increasing concentrations of phosphate, fractions with maximal ^{125}I-Aß binding activity were identified, pooled and applied to preparative nonreduced SDS-PAGE (10%). Gels were stained with Coomassie blue, and identical lanes of the gel were either cut into 2 mm slices, protein was eluted and studied

for Aß binding activity as above, or slices of the gel were subjected to N-terminal sequence analysis (Hori et al., 1995).

A DNA fragment coding for human sRAGE was obtained from a lung library by PCR using human recombinant soluble RAGE was prepared from the human RAGE cDNA (primers: 5'GATGGCAGCCGGAACAGCAGTT3'(Seq. I.D. No. 4) and 5'CTCAAGTTCCCAGCCCTGATCCTCC3') (Seq. I.D. No. 5) and cloned into the pBacPAK8 vector (Clontech) for expression of recombinant protein as described (Hori et al., 1995). Recombinant sRAGE was purified to homogeneity from Sf9 media by chromatography on SP Sepharose fast flow and Superdex 200PG chromatography as described (Hori et al., 1995) (Pharmacia) equilibrated with sodium phosphate (20 mM using a baculovirus expression system as described previously for amphoterin (Hori et al., 1995). Antibody was prepared to human recombinant sRAGE by immunizing rabbits according to standard procedures (Vaitukaitis, 1981), and purified IgG was characterized as monospecific by immunoblotting and ELISA. A DNA fragment coding for the first three immunoglobulin-like domains of VCAM-1 was obtained from a lung cDNA library using PCR (primers: 5'GAAATGCCTGTGAAGATGGTCG3'(Seq. I.D. No. 6) and 5'CTCATTGAACACTAATTCCACTTC3')(Seq. I.D. No. 7), expressed and purified as above for sRAGE except that Q Sepharose (Pharmacia) was used (20 mM, pH 8.0).

Aß-RAGE induced cellular oxidant stress and cytotoxicity. Evidence of Aß-induced cellular perturbation was studied by evaluating generation of TBARS (Dennery et al., 1990) and reduction of MTT (Promega, Madison WI). In addition, cultures were examined using an inverted Olympus PX-10 microscope. Electrophoretic mobility shift assays (EMSA) were performed to assess nuclear translocation of NF-kB (Yan et al., 1995). Extracts of nuclear protein were derived

from cultured cells, as indicated, and reacted with a consensus ^{32}P-labelled human NF-kB probe.

Immunohistologic studies were performed on paraffin sections of formalin-fixed temporal lobe from AD and age-matched controls (Yan et al., 1995). Sections were first stained with either rabbit anti-human RAGE IgG (20 μg/ml), rabbit anti-human heme oxygenase type I (StressGen, Vancouver Canada), or rabbit anti-human p50 IgG (StressGen), and sites of binding of primary antibody were visualized using the avidin/biotin alkaline phosphatase method. Double staining of these sections for Aß was performed using murine monoclonal anti-human Aß IgG (5 μg/ml), followed by peroxidase-conjugated goat anti-mouse IgG. Nonimmune rabbit or mouse IgG were used as controls.

Example 2 : A Receptor Mediating Activation of Microglia by Amyloid-β peptide.

Infiltration of ß-amyloid plaques by reactive microglia in Alzheimer's disease Itagaki et al., 1989; Dickson et al., 1993; and McGeer et al., 1993) as well as ß-amyloid induced activation of microglia in vitro (Davis et al., 1992; Klegeris et al., 1994; Meda et al., 1995; and Araujo and Cotman, 1992), suggests that ß-amyloid-microglial interaction involves specific pathways. RAGE, an immunoglobulin superfamily receptor previously shown to engage advanced glycation endproducts (Schmidt et al., 1992; and Neeper et al., 1992), serves as a specific binding site for ß-amyloid (1-40 and 25-35), and that its expression is enhanced in reactive microglia in Alzheimer's brain. RAGE has a central role in ß-amyloid-induced microglial activation: blockade of RAGE prevents binding of ^{125}I-ß-amyloid(1-40) to the cell surface; induction of microglial migration by soluble ß-amyloid and arrest of microglia by immobilized ß-amyloid are mediated by RAGE; and stimulation of microglial expression of tumor necrosis

factor-a and activation of NF-kB by ß-amyloid is due to binding to RAGE. These data suggest that blockade of ß-amyloid-RAGE-dependent microglial activation might prevent triggering of chronic inflammatory cascades in Alzheimer's brain.

The association of activated microglia with amyloid plaques in the brain of patients with Alzheimer's disease (AD) is consistent with an ongoing immune/inflammatory response, as evidenced by microglial expression of the class II major histocompatibility antigen HLA-DR, and cytokines, such as Interleukins 1 and 6 (Dickson et al., 1993; and McGeer et al., 1993). That changes in microglial activation might be a direct consequence of ß-amyloid (Aß)-microglial interaction is supported by the observation that soluble Aß(1-40 and 25-35) promotes microglial chemotaxis, as well as TNF, Interleukin 1ß, and basic fibroblast growth factor expression and nitrite production (Davis et al., 1992; Klegeris et al., 1994; Meda et al., 1995; and Araujo and Cotman, 1992). Recently it has been found that RAGE, Receptor for Advanced Glycation Endproducts (Schmidt et al., 1992; and Neeper et al., 1992), binds Aß(1-40 and 25-35) in a specific, dose-dependent, and saturable manner (K_d μ55 nM); and Aß-RAGE interaction mediates, at least in part, neuronal cytotoxicity and oxidant stress due to Aß (Yan et al., 1996). As RAGE is expressed by mononuclear phagocytes (Schmidt et al., 1993), we sought its presence in activated microglia in AD brain. Microglia, identified by staining with CD68 (Fig. 8A), proximate to a senile plaque, show strong expression of RAGE antigen (Fig. 8B).

Radioligand binding studies with BV-2 cells demonstrated dose-dependent binding of ^{125}I-Aß(1-40) with Kd ≈30nM (Fig. 9A). Blockade of RAGE, with either excess sRAGE or anti-RAGE IgG, prevented binding of ^{125}I-Aß to microglia, although soluble vascular cell adhesion molecule-1 (another

immunoglobulin superfamily molecule) or nonimmune rabbit IgG was without effect (Fig. 9B).

As RAGE appeared to have a central role in tethering Aß to microglia, it was considered whether Aß-RAGE interaction might underlie Aß-induced migration of microglia. Using BV-2 cells, addition of Aß (1-40 or 25-35) to microchemotaxis chambers resulted in directional cell migration, dependent on a concentration gradient of the stimulus from upper to lower chamber (Fig. 10A). Cell migration was prevented by addition of anti-RAGE $F(ab')_2$, whereas this reagent had no affect on motility of BV-2 cells following addition of formylated peptide (formyl-methionyl-leucinyl phenylalanine, fMLP)(Fig. 10B). In contrast, nonimmune rabbit IgG was without effect on Aß-induced migration of BV-2 cells. Because of the known accumulation of microglia approximate to deposits of Aß, it was possible that, in contrast to soluble Aß, immobilized Aß might block microglial migration. To test this concept, either Aß or albumin was adsorbed to the upper surface of chemotaxis chamber membranes and BV-2 cells were added; cell migration across Aß-coated membranes was diminished in response to a range of fMLP concentrations added to the lower compartment, whereas albumin-coated membranes allowed free passage of cells (Fig. 10C), comparable to untreated membranes. Blockade of RAGE with anti-RAGE $F(ab')_2$ enhanced BV-2 migration across Aß-coated membranes to levels observed in membranes coated with albumin, whereas nonimmune $F(ab')_2$ was without effect. Similar results were observed in a more limited number of studies on microglia isolated from rat brain). These data indicate that soluble Aß induces microglial migration whereas immobilized Aß arrests microglial migration, in each case due to Aß engagement of RAGE.

Activation of microglia, potentially producing cytotoxic

cytokines and other species (Dickson et al., 1993; McGeer et al., 1993; Meda et al., 1995; and Araujo et al., 1992), may be an important pathway to neuronal damage in AD. Of these, the ability of Aß to stimulate microglial expression of tumor necrosis factor-a (TNFα) (Meda et al., 1995) may be especially significant, as TNFa could recruit additional host effector mechanisms. BV-2 cells incubated with Aß demonstrated induction of TNFα transcripts by PCR (Fig. 11A), followed by elaboration of TNFα into culture supernatants (Fig. 11B). Blockade of RAGE, with anti-RAGE $F(ab')_2$, prevented the appearance of TNFα mRNA and diminished TNFα antigen to levels seen in untreated cells, whereas nonimmune $F(ab')_2$. A mechanism through which Aß and other cytokines influence cellular activation is by inducing nuclear translocation of the transcription factor NF-kB, often utilizing an oxidant-mediated pathway (Schreck et al., 1991). Incubation of BV-2 cells with microglia induced NF-kB activation which was suppressed by blocking RAGE with anti-RAGE $F(ab')_2$ (Fig. 11C) or the antioxidant probucol. Nonimmune $F(ab')_2$ was without effect.

These data show that RAGE has a pivotal role in the interaction of Aß with microglial cells. Soluble Aß binds RAGE and induces microglial migration along a concentration gradient, likely to end at a deposit of insoluble Aß. Immobilized Aß (prepared as above or after allowing time for fibrils of Aß to form) prevents cell migration, permitting microglia to undergo the sustained activation which may underlie a chronic inflammatory component in AD(Rogers et al., 1993; and Breitner et al., 1994). Indeed, in some studies it was found that immobilized Aß does induce microglial activation, and the time course for TNF production is more prolonged than that observed following exposure of microglia to soluble Aß. Although these data indicate that RAGE is important in mediating microglial binding and subsequent cellular activation following

exposure to Aß, in view of the number of different polypeptides with which Aß has been shown to interact (Iversen et al., 1995), it is likely that other proteins will also be identified which participate with RAGE in Aß-mediated cellular activation. In this context, these studies have emphasized the potential of Aß-RAGE interaction to activate microglia, accelerating events leading to neurodegeneration. However, RAGE processing of Aß (i.e., endocytosis and degradation), under conditions where substantial amounts of Aß are present probably represents a protective mechanism gone awry: it is possible that small amounts of Aß might be endocytosed and degraded by RAGE-dependent mechanisms, whereas larger amounts of peptide might overwhelm this mechanism and result in Aß-RAGE-induced cellular activation. These studies complement the recent observation that RAGE is expressed at higher levels in neurons approximate to Aß deposits (Yan et al., 1996). RAGE appears to mediate oxidant stress and neurotoxicity, as shown by their attenuation following blockade of the receptor (Yan et al., 1996). The possibility that Aß in AD is subject to nonenzymatic glycation with formation of advanced glycation endproducts (the latter were the first recognized ligands of RAGE) provides an additional mechanism for enhancing its interaction with RAGE (Vitek et al., 1994). Taken together, these observations suggest that increased expression of RAGE in AD brain may have a central role in Aß-mediated microglial activation, as well as neuronal perturbation. Thus, interfering with Aß-RAGE interaction might delay the development of subsequent cellular events resulting in neuronal dysfunction.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Arg Gly Asp Ser
1

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1 5 10 15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
20 25 30

Gly Leu Met Val Gly Gly Val Val
35 40

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATGGCAGCC GGAACAGCAG TT
22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCAAGTTCC CAGCCCTGAT CCTCC
25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAAATGCCTG TGAAGATGGT CG
22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCATTGAAC ACTAATTCCA CTTC
24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Gln Asn Ile Thr Ala Arg Ile Gly Lys Pro Leu Val Leu Asn Cys
1 5 10 15

Lys Gly Ala Pro Lys Lys Pro Pro Gln Gln Leu Glu Trp Lys
20 25 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asp Gln Xaa Ile Thr Ala Arg Ile Gly Lys Pro Leu Val Leu Asn Xaa
1 5 10 15

Lys Gly Ala Pro Lys Lys Pro Pro Gln Gln Leu Glu Trp Lys
20 25 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Gln Xaa Ile Thr Ala Arg Ile Gly Lys Pro Leu Val Leu Asn Xaa
1 5 10 15

Lys Gly Ala Pro
20

What is claimed is:

1. A method for inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises contacting the cell with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit interaction of the amyloid-β peptide with the receptor for advanced glycation end product on the surface of the cell.

2. The method of claim 1, wherein the cell is a neuronal cell, an endothelial cell, a glial cell, a microglial cell, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, an endothelial cell, a tumor cell, or a stem cell.

3. The method of claim 1, wherein the agent is a peptide, a peptidomimetic, a nucleic acid or a small molecule.

4. The method of claim 3, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2).

5. The method of claim 3, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

6. The method of claim 1, wherein the agent is soluble receptor for advanced glycation end product.

7. The method of claim 1, wherein the agent is a soluble

extracellular portion of a receptor for advanced glycation end product.

8. The method of claim 1, wherein the agent is an antibody or portion thereof.

9. The method of claim 8, wherein the antibody is capable of specifically binding to the receptor for advanced glycation endproduct.

10. The method of claim 8, wherein the antibody is a monoclonal antibody.

11. The method of claim 8, wherein the antibody is a polyclonal antibody.

12. The method of claim 8, wherein the portion of the antibody comprises a Fab.

13. The method of claim 8, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

14. The method of claim 1, wherein the agent is capable of specifically binding to the amyloid-β peptide.

15. The method of claim 14, wherein the agent binds to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

16. A method for inhibiting degeneration of a neuronal cell which comprises contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the neuronal cell according to the method of claim 1.

17. A method for inhibiting formation of an amyloid-β peptide fibril on a cell which comprises contacting the cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the cell according to the method of claim 1.

18. A method for inhibiting extracellular assembly of an amyloid-β peptide into a fibril which comprises contacting the amyloid-β peptide with an agent capable of inhibiting interaction of an amyloid-β peptide with another amyloid-β peptide according to the method of claim 1.

19. A method for inhibiting aggregation of amyloid-β peptide on the surface of a cell which comprises contacting the amyloid-β peptide with an agent capable of inhibiting interaction of the amyloid-β peptide with a receptor for advanced glycation end product according to the method of claim 1.

20. A method for inhibiting aggregation of amyloid-β peptide on the surface of a cell which comprises contacting the receptor for advanced glycation end product with an agent capable of inhibiting interaction of the amyloid-β peptide with the receptor for advanced glycation end product according to the method of claim 1.

21. A method for inhibiting infiltration of a microglial cell into senile plaques which comprises contacting the microglial cell with an agent capable of inhibiting interaction of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of the microglial cell according to the method of claim 1.

22. A method for inhibiting activation of a microglial cell by an amyloid-β peptide which comprises contacting the microglial cell with an agent capable of inhibiting interaction of the amyloid-β peptide with a receptor for advanced glycation end product on the surface of the microglial cell according to the method of claim 1.

23. The method of claim 22, wherein the inhibition of activation comprises decreased production of cytokines by the microglial cell.

24. The method of claim 1, wherein the interaction is binding of the amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

25. A method for treating a subject with a condition associated with interaction of an amyloid-β peptide with a receptor for advanced glycation end product on a cell, which comprises administering to the subject an agent capable of inhibiting the interaction of the amyloid-β peptide with the receptor for advanced glycation end product, the agent being present in an amount effective to inhibit the amyloid-β peptide interaction with the receptor for advanced glycation end product on the cell thereby treating the subject.

26. The method of claim 25, wherein the condition is diabetes, Alzheimer's Disease, senility, renal failure, hyperlipidemic atherosclerosis, neuronal cytotoxicity, Down's syndrome, dementia associated with head trauma, amyotrophic lateral sclerosis, multiple sclerosis or neuronal degeneration.

27. The method of claim 25, wherein the subject is a

mammal.

28. The method of claim 27, wherein the mammal is a human.

29. The method of claim 25, wherein the administration comprises intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

30. The method of claim 25, wherein the agent is soluble receptor for advanced glycation end product.

31. The method of claim 25, wherein the agent is a soluble extracellular portion of a receptor for advanced glycation end product.

32. The method of claim 25, wherein the agent is a peptide, a peptidomimetic, a nucleic acid or a small molecule.

33. The method of claim 32, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2).

34. The method of claim 32, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

35. The method of claim 25, wherein the agent is an antibody or portion thereof.

36. The method of claim 35, wherein the antibody is capable of specifically binding to the receptor for advanced

glycation endproduct.

37. The method of claim 35, wherein the antibody is a monoclonal antibody.

38. The method of claim 35, wherein the antibody is a polyclonal antibody.

39. The method of claim 35, wherein the portion of the antibody comprises a Fab.

40. The method of claim 35, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

41. The method of claim 25, wherein the agent is capable of specifically binding to the amyloid-β peptide.

42. The method of claim 41, wherein the agent binds to the amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

43. The method of claim 25, wherein the condition is associated with degeneration of a neuronal cell in the subject.

44. The method of claim 25, wherein the condition is associated with formation of an amyloid-β peptide fibril.

45. The method of claim 25, wherein the condition is associated with aggregation of amyloid-β peptide.

46. The method of claim 25, wherein the condition is associated with infiltration of a microglial cell into a senile plaque.

47. The method of claim 25, wherein the condition is associated with activation of a microglial cell by an amyloid-β peptide.

48. The method of claim 47, wherein the activation comprises production of cytokines by the microglial cell.

49. A method for evaluating the ability of an agent to inhibit binding of an amyloid-β peptide with a receptor for advanced glycation end product on the surface of a cell which comprises:

(a) contacting the cell with the agent and amyloid-β peptide;

(b) determining the amount of amyloid-β peptide bound to the cell, and

(c) comparing the amount of bound amyloid-β peptide determined in step (b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-β peptide to the receptor for advanced glycation end product on the surface of the cell.

50. The method of claim 49, wherein the cell is contacted with the agent and the amyloid-β peptide simultaneously.

51. The method of claim 49, wherein the cell is contacted with the amyloid-β peptide and the agent.

52. The method of claim 49, wherein the cell is a neuronal cell, an endothelial cell, a glial cell, a microglial

cell, a smooth muscle cell, a somatic cell, a bone marrow cell, a liver cell, an intestinal cell, a germ cell, a myocyte, a mononuclear phagocyte, an endothelial cell, a tumor cell, or a stem cell.

53. The method of claim 49, wherein the agent is a peptide, a peptidomimetic, a nucleic acid or a small molecule.

54. The method of claim 53, wherein the peptide comprises at least a portion of the sequence -Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val- (Seq. I.D. No. 2).

55. The method of claim 53, wherein the peptide comprises at least a portion of the sequence -Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met- (Seq. I.D. No. 3).

56. The method of claim 49, wherein the agent is soluble receptor for advanced glycation end product.

57. The method of claim 49, wherein the agent is a soluble extracellular portion of receptor for advanced glycation end product.

58. The method of claim 49, wherein the agent is an antibody or portion thereof.

59. The method of claim 58, wherein the antibody is capable of specifically binding to the receptor for advanced glycation endproduct.

60. The method of claim 58, wherein the antibody is a monoclonal antibody.

61. The method of claim 58, wherein the antibody is a polyclonal antibody.

62. The method of claim 58, wherein the portion of the antibody comprises a Fab.

63. The method of claim 58, wherein the portion of the antibody comprises a complementarity determining region or a variable region.

64. The method of claim 49, wherein the agent is capable of specifically binding to the amyloid-β peptide.

65. The method of claim 64, wherein the agent binds to amyloid-β peptide at the site where the receptor for advanced glycation end product interacts.

66. The method of claim 49, wherein the agent is a soluble extracellular portion of a receptor for advanced glycation end product.

67. The method of claim 49, wherein the agent is bound to a solid support.

68. The method of claim 49, wherein the agent is expressed on the surface of a cell.

69. A method for inhibiting activation of an NF-κB gene in a cell which comprises contacting the cell with an agent which is capable of inhibiting interaction of amyloid-β peptide with a receptor for advanced glycation endproduct on the cell, thus inhibiting activation of NF-κB in the cell.

70. A pharmaceutical composition which comprises an agent capable of inhibiting interaction of amyloid-β peptide

with a receptor for advanced glycation endproduct and a pharmaceutically acceptable carrier.

71. The pharmaceutical composition of claim 70, wherein the carrier is a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

FIG. 1A FIG. 1B FIG. 1C

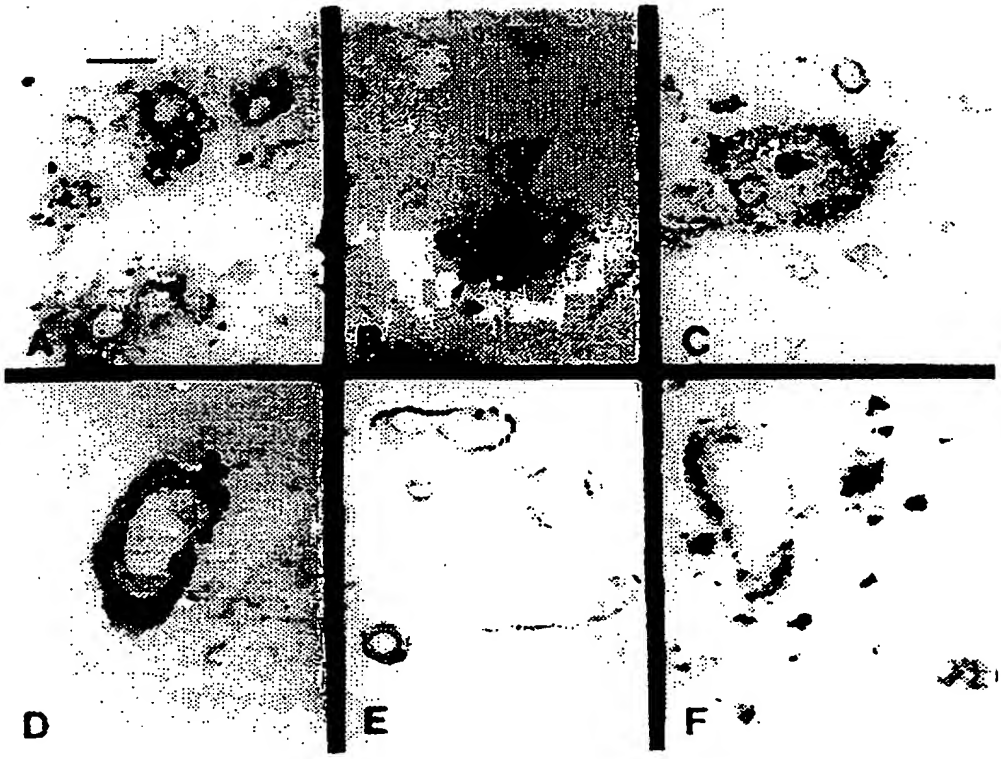

FIG. 1D FIG. 1E FIG. 1F

FIG. 1G

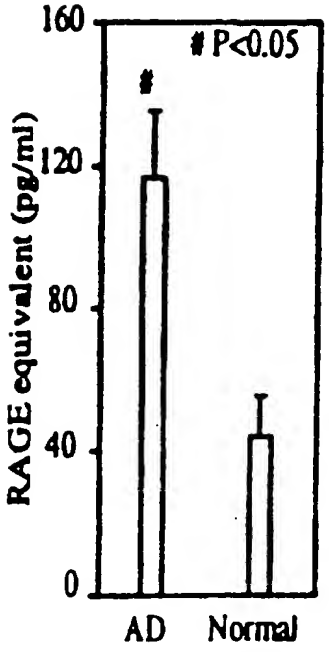

FIG. 2A

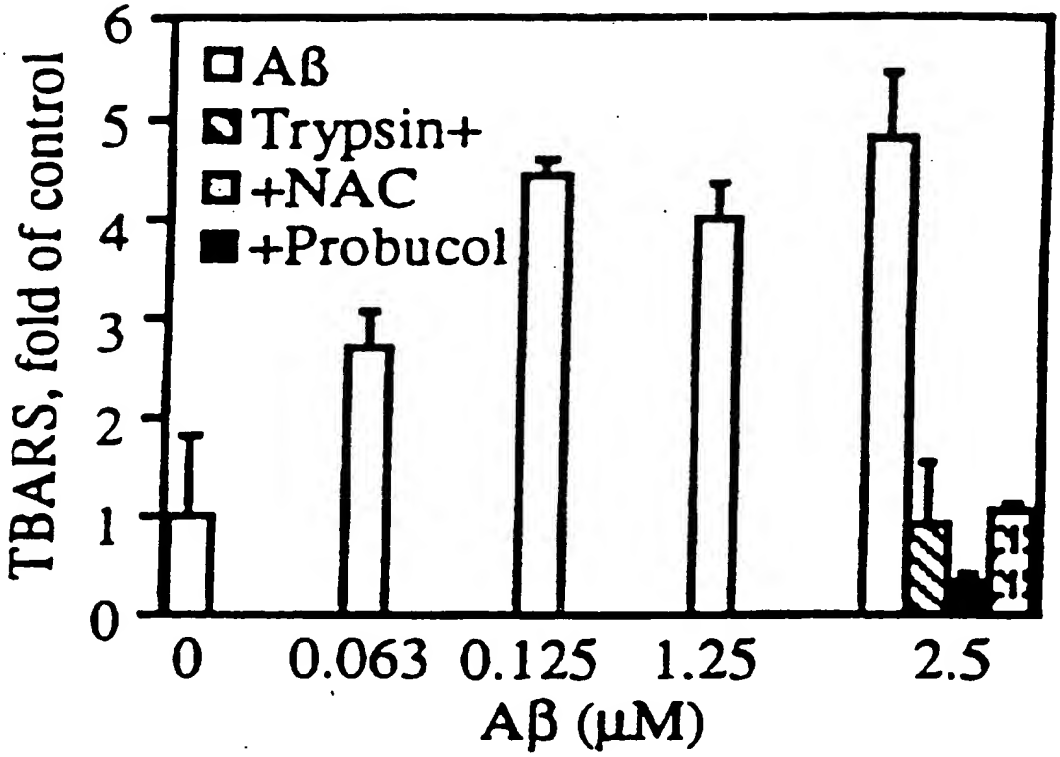
TBARS, fold of control
0
1
2
3
4
5
6
Aß
Trypsin+
+NAC
+Probucol
0
0.063
0.125
1.25
2.5
Aβ (μM)

FIG. 2B

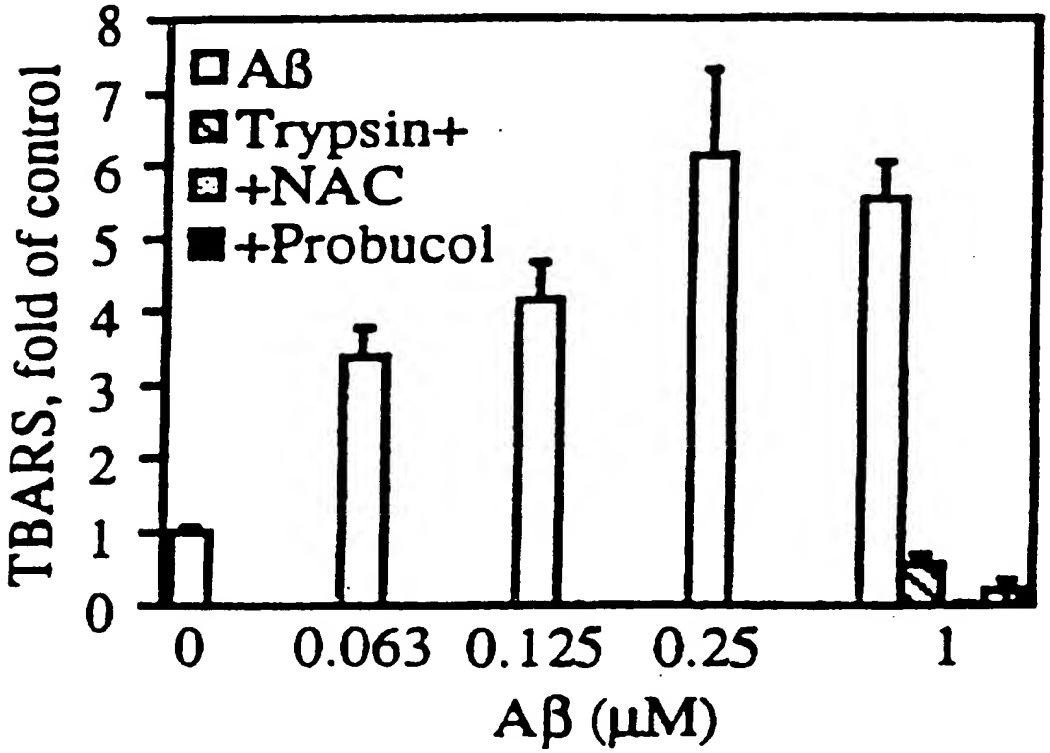
TBARS, fold of control
0
1
2
3
4
5
6
7
8
Aß
Trypsin+
+NAC
+Probucol
0
0.063
0.125
0.25
1
Aβ (μM)

FIG. 2C-1

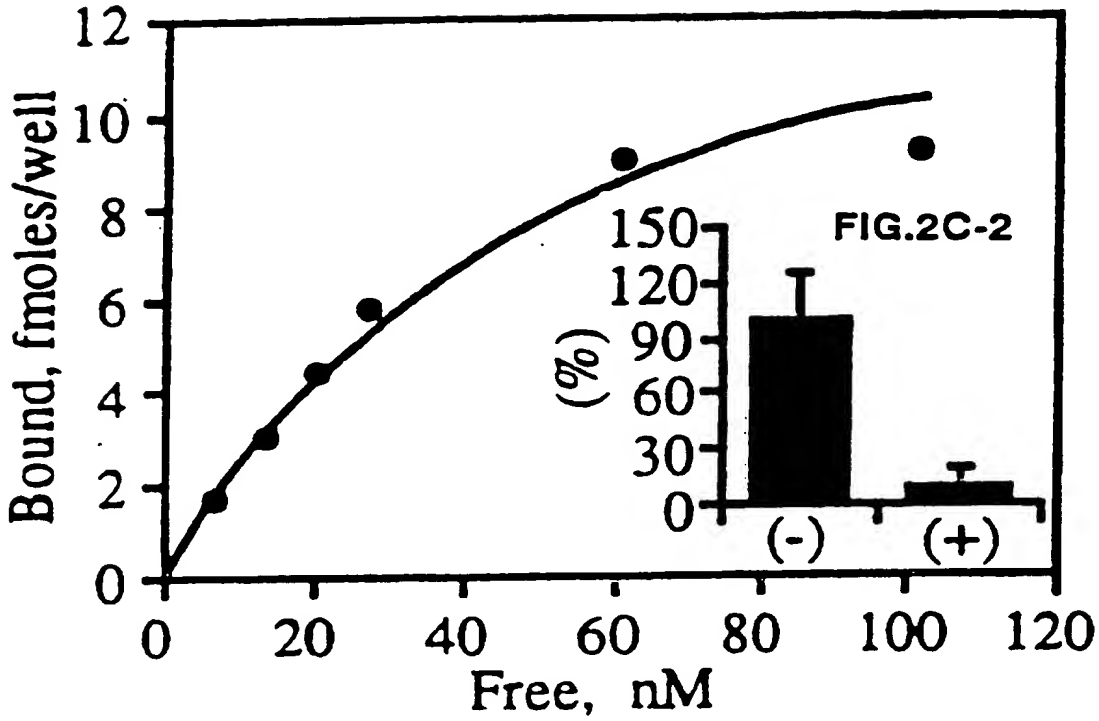

FIG. 2D-1

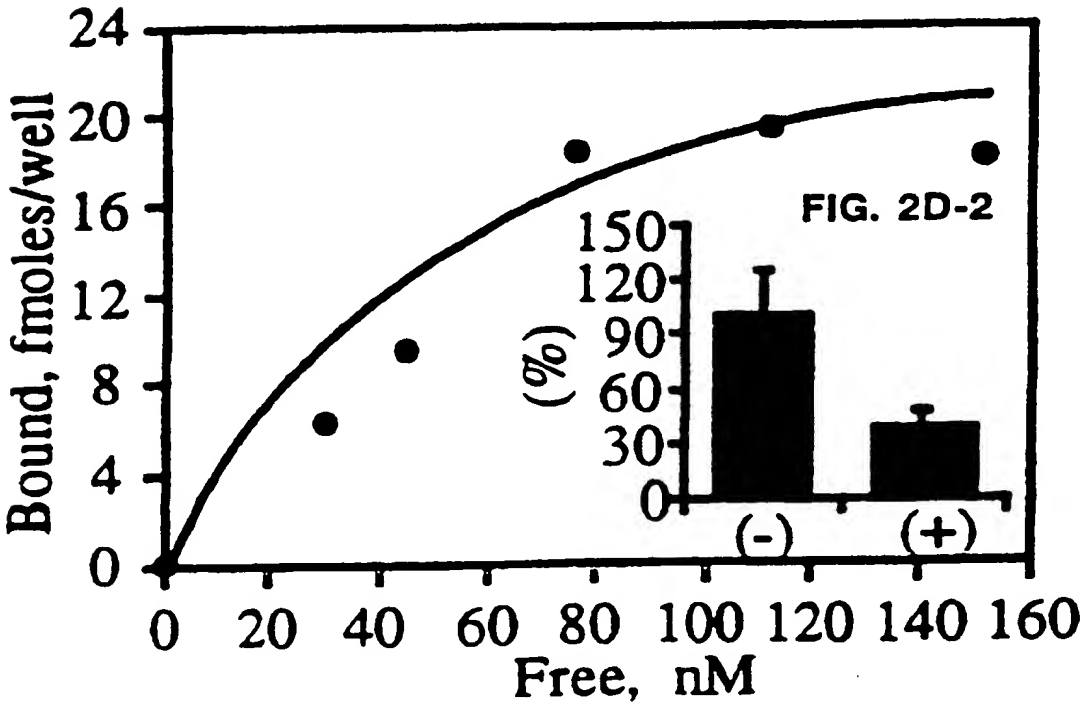

FIG. 2E

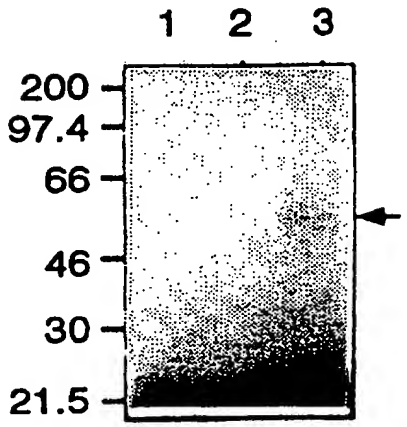

FIG. 2F

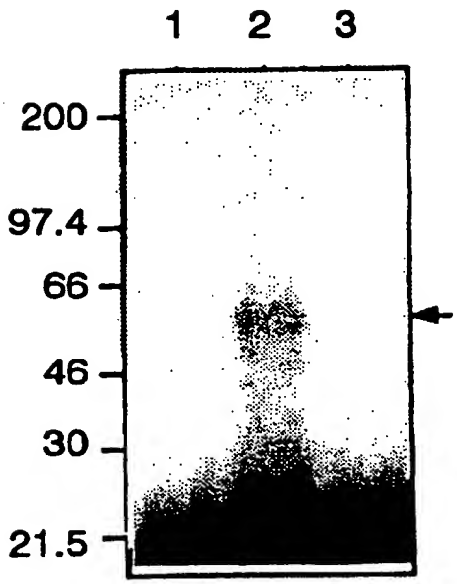

FIG. 3A

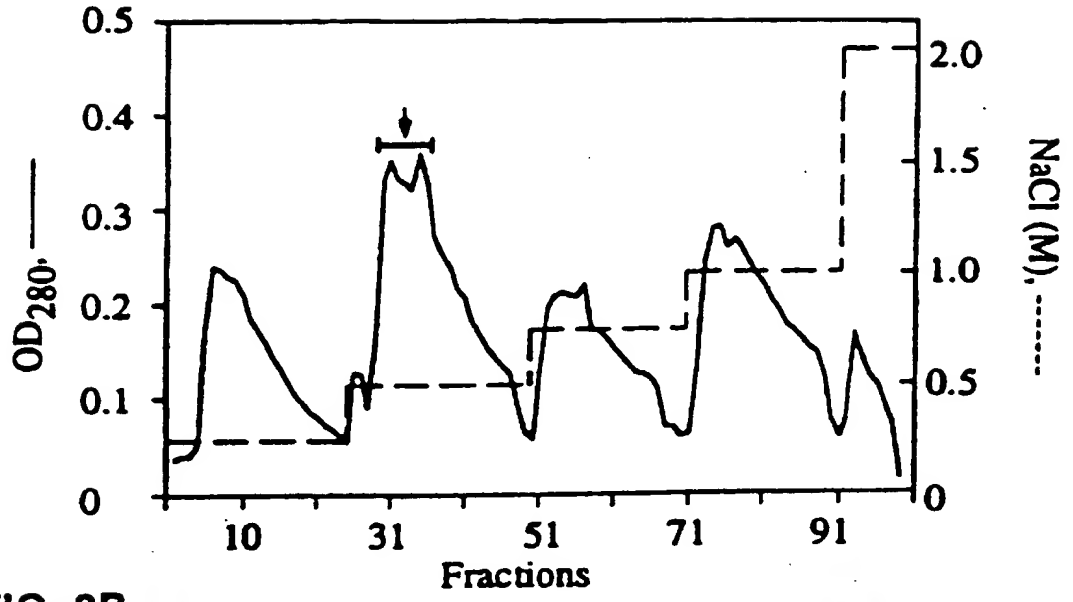
0.5
0.4
0.3
0.2
0.1
0
OD_{280}, ——
2.0
1.5
1.0
0.5
0
NaCl (M), ------
10
31
51
71
91
Fractions

FIG. 3B

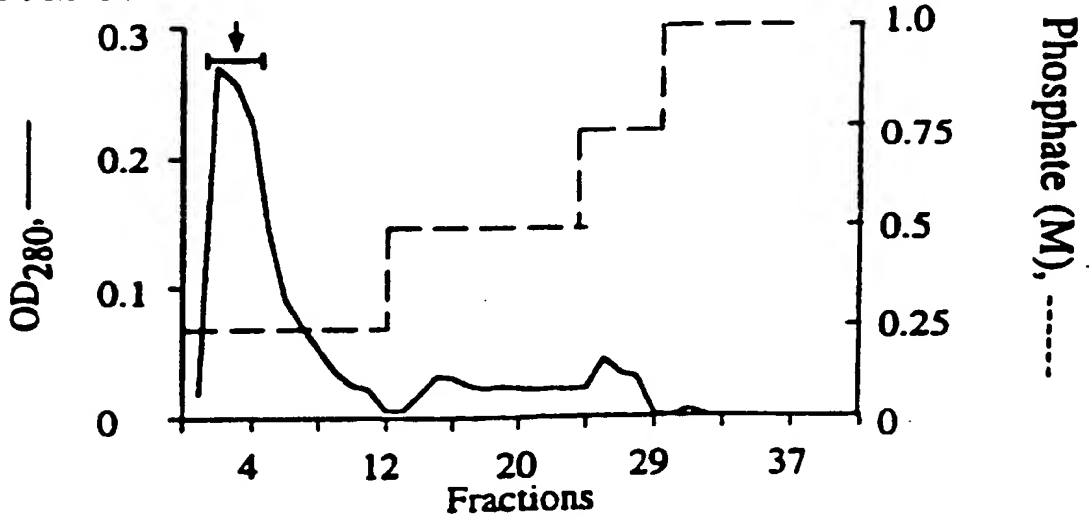
0.3
0.2
0.1
0
OD_{280}, ——
1.0
0.75
0.5
0.25
0
Phosphate (M), ------
4
12
20
29
37
Fractions

FIG. 3C

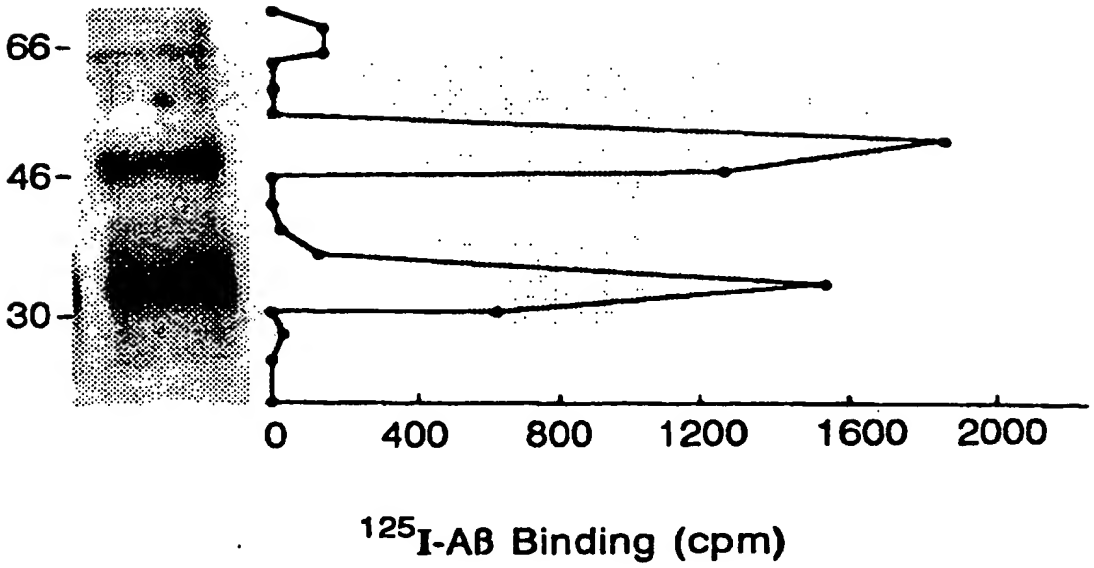

FIG. 4A

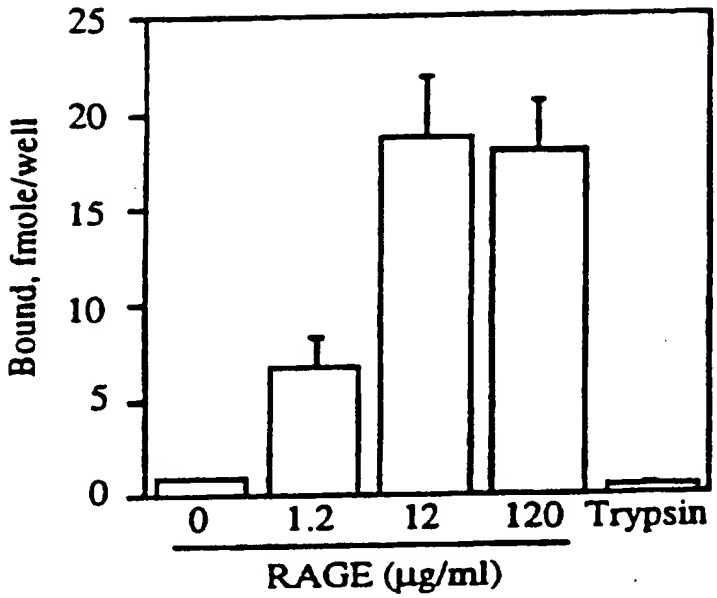
25
20
15
10
5
0
Bound, fmole/well
0
1.2
12
120
Trypsin
RAGE (μg/ml)

FIG. 4B

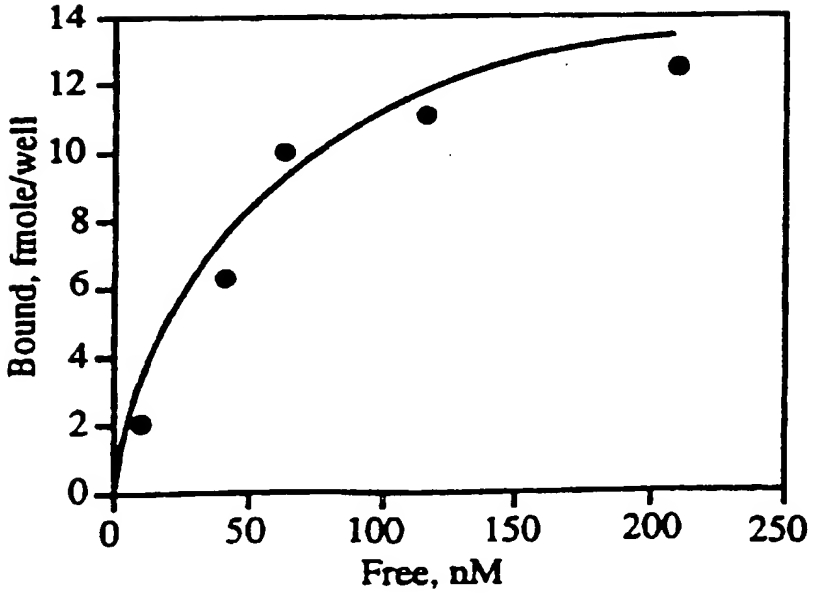
14
12
10
8
6
4
2
0
Bound, fmole/well
0
50
100
150
200
250
Free, nM

FIG. 4C

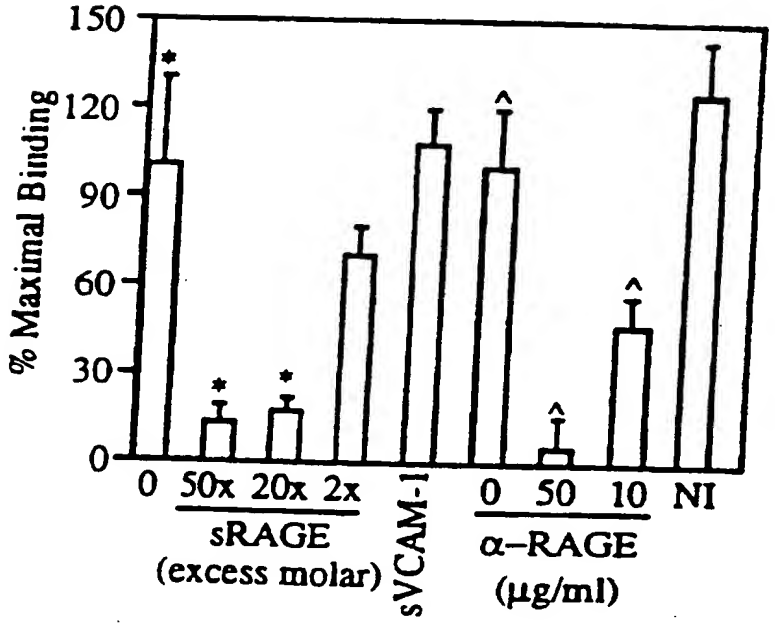
150
120
90
60
30
0
% Maximal Binding
0 50x 20x 2x
sRAGE
(excess molar)
sVCAM-1
0 50 10 NI
α–RAGE
(μg/ml)

FIG. 4D

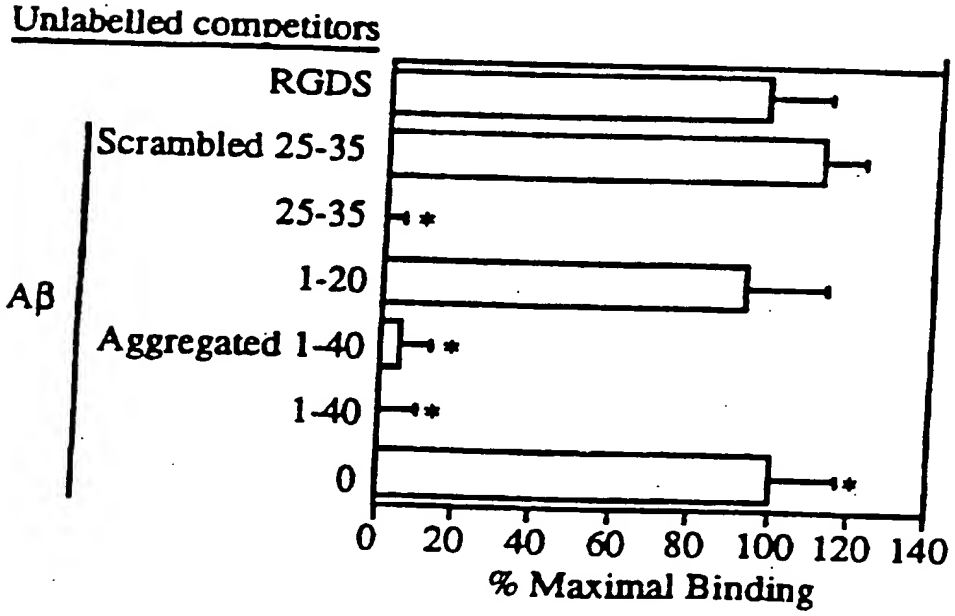
Unlabelled competitors
RGDS
Scrambled 25-35
25-35
1-20
Aβ
Aggregated 1-40
1-40
0
0 20 40 60 80 100 120 140
% Maximal Binding

FIG. 4E

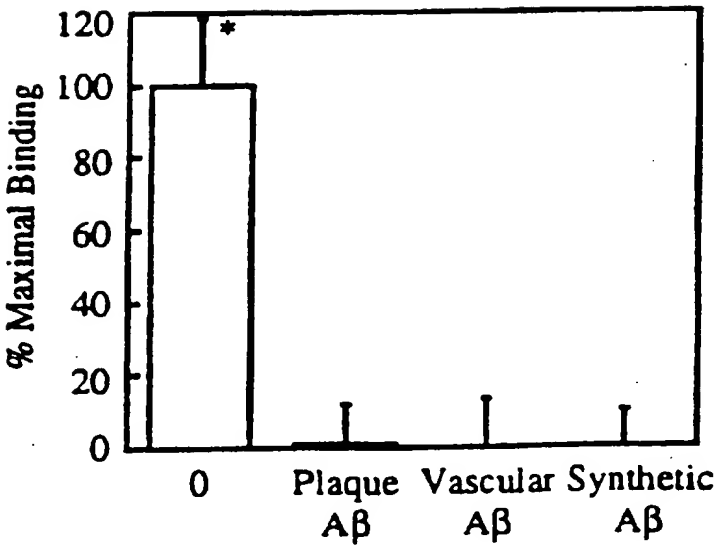
% Maximal Binding
120
100
80
60
40
20
0
*
0
Plaque Aβ
Vascular Aβ
Synthetic Aβ

FIG. 5A

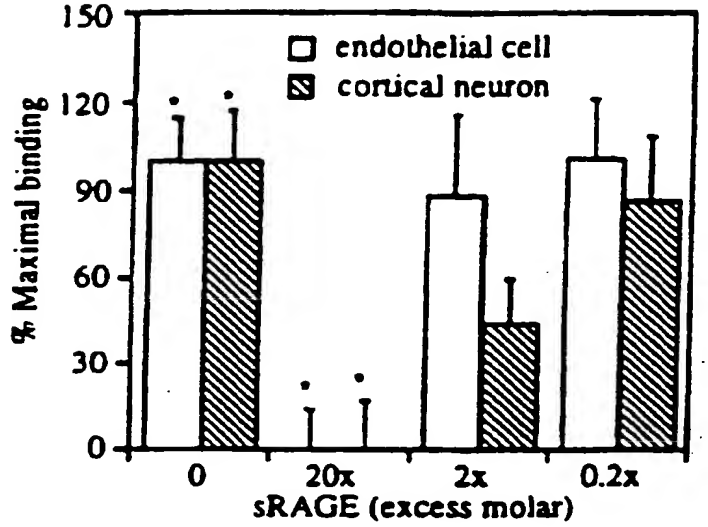

FIG. 5B

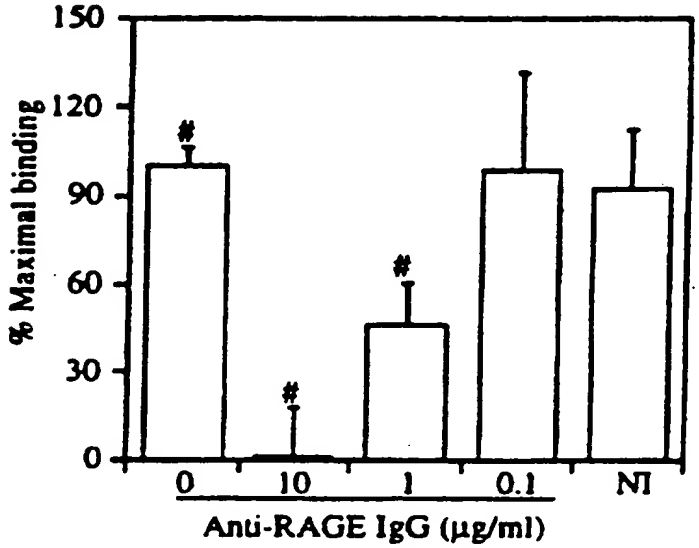

FIG. 5C

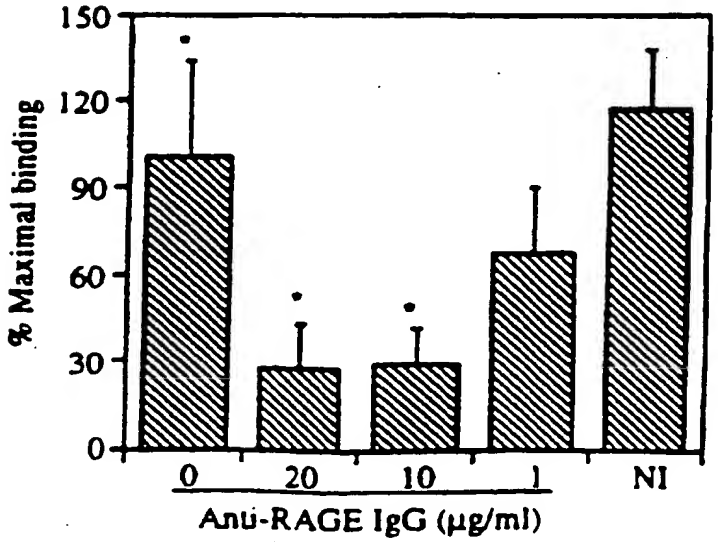

FIG. 6A

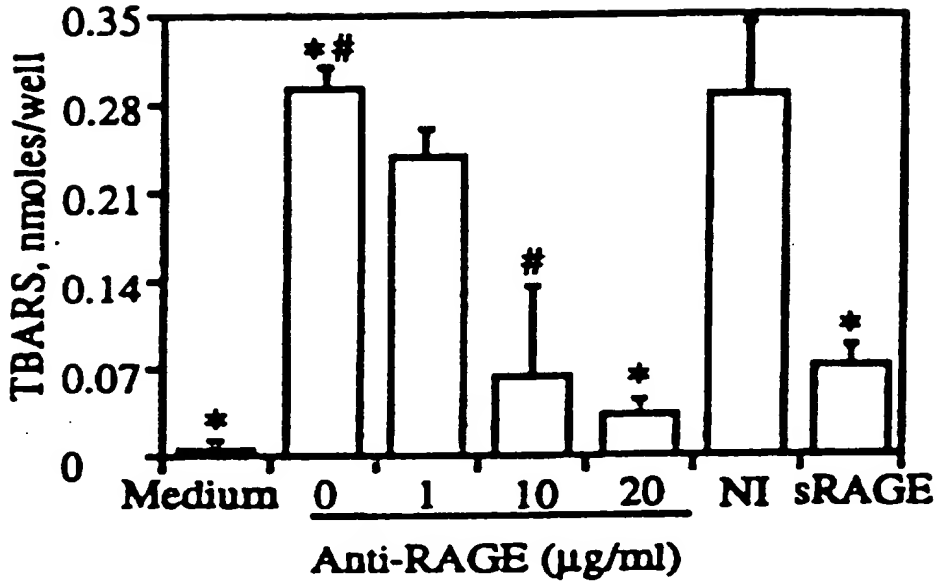
0.35
0.28
0.21
0.14
0.07
0
TBARS, nmoles/well
*#
#
*
*
*
Medium
0
1
10
20
NI
sRAGE
Anti-RAGE (µg/ml)

FIG. 6B

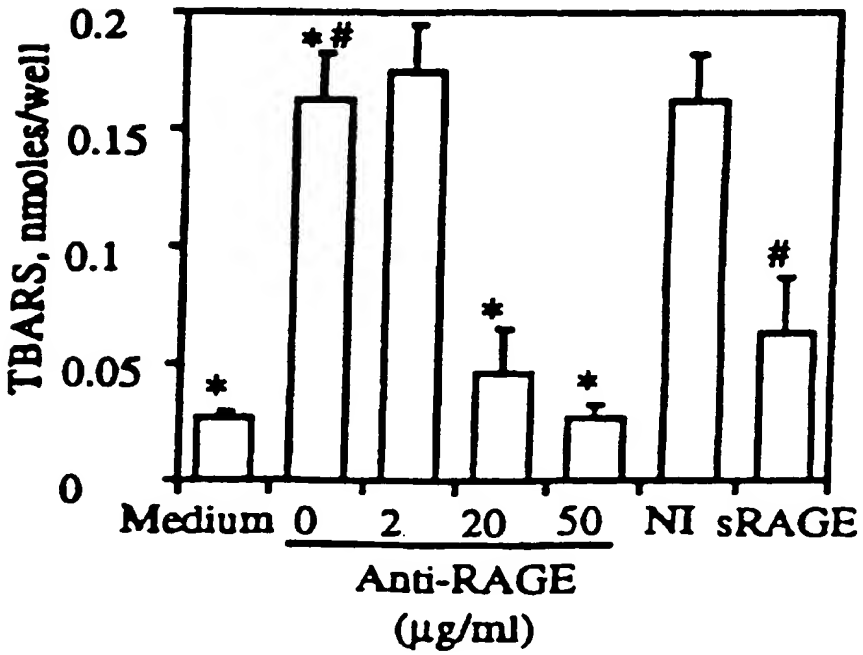
0.2
0.15
0.1
0.05
0
TBARS, nmoles/well
*#
*
*
*
#
Medium
0
2
20
50
NI
sRAGE
Anti-RAGE
(µg/ml)

FIG. 6C

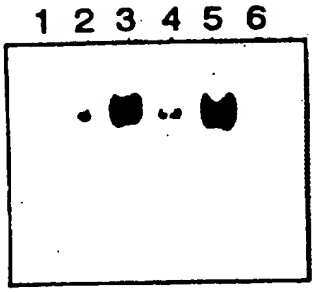

FIG. 6D

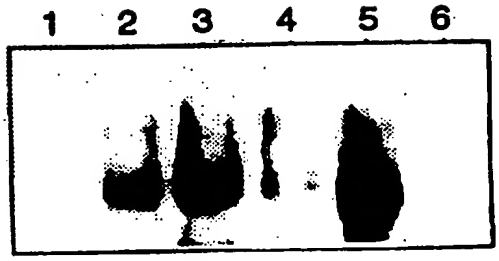

FIG. 7A

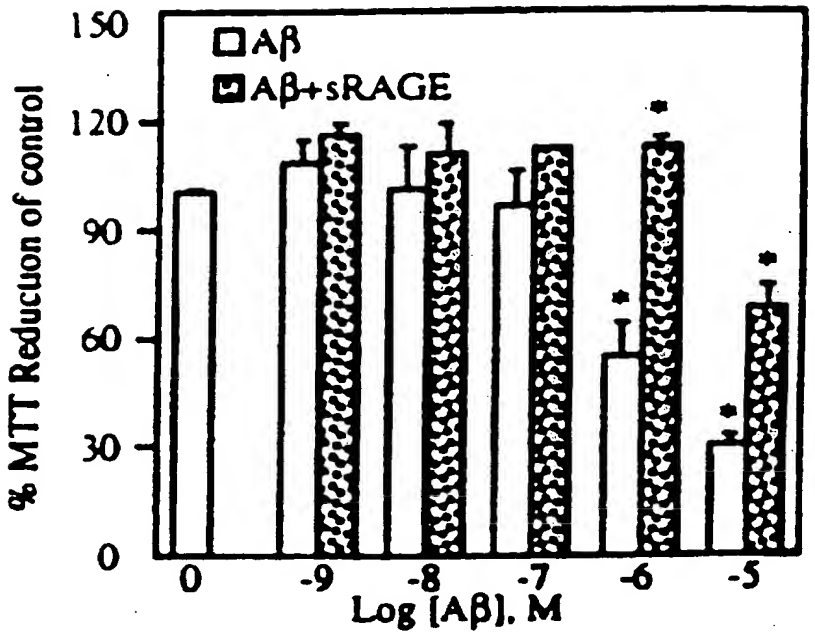
Aβ
Aβ+sRAGE
% MTT Reduction of control
150
120
90
60
30
0
0
-9
-8
-7
-6
-5
Log [Aβ], M

FIG. 7B

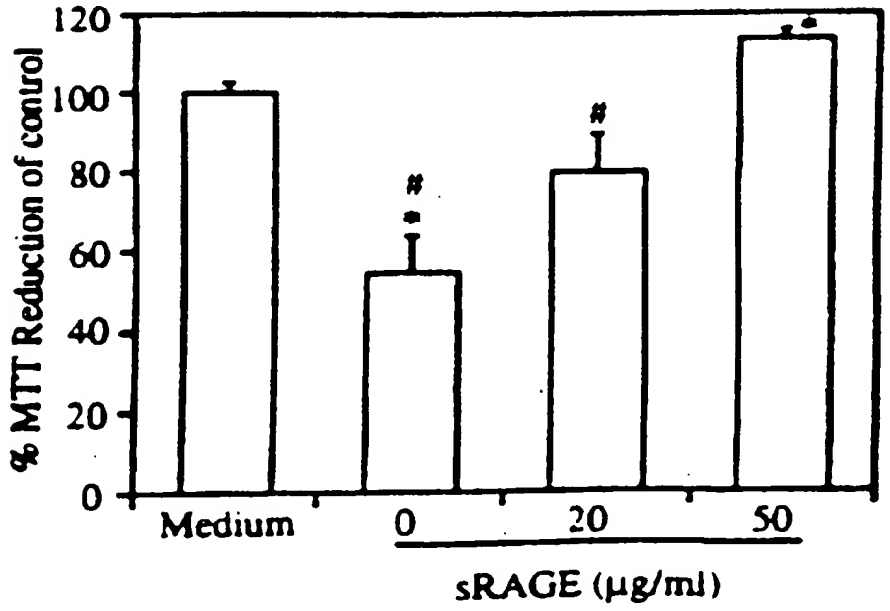
% MTT Reduction of control
120
100
80
60
40
20
0
Medium
0
20
50
sRAGE (μg/ml)

FIG. 7C

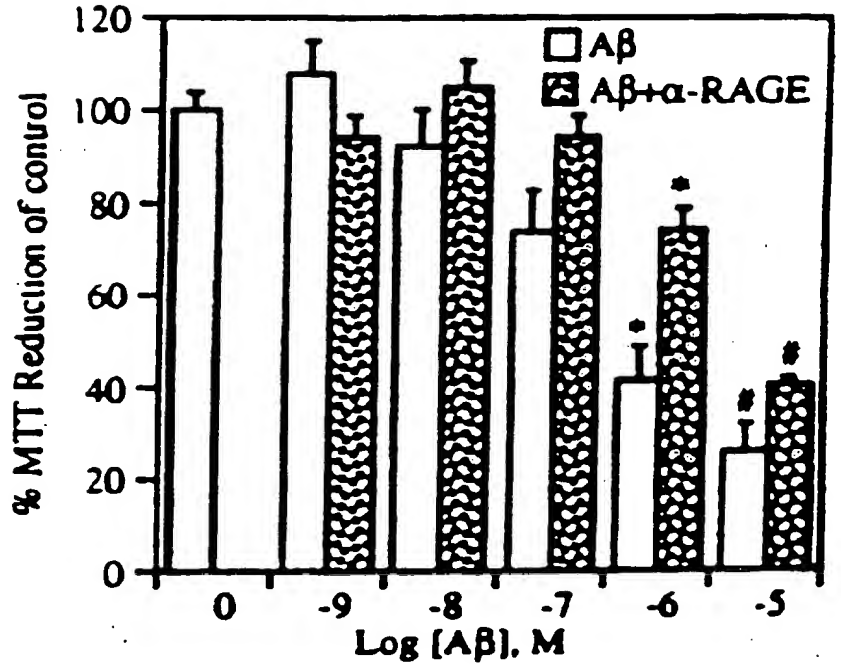
120
100
80
60
40
20
0
% MTT Reduction of control
Aβ
Aβ+α-RAGE
0
-9
-8
-7
-6
-5
Log [Aβ], M

FIG. 7D

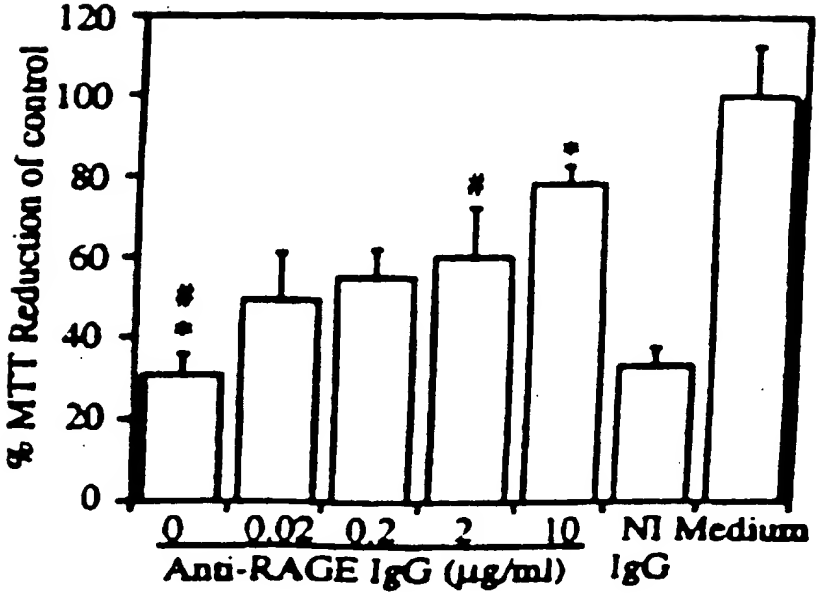
120
100
80
60
40
20
0
% MTT Reduction of control
0
0.02
0.2
2
10
NI Medium
Anti-RAGE IgG (μg/ml)
IgG

FIG. 7E

FIG. 7F

FIG. 7G

FIG. 7H

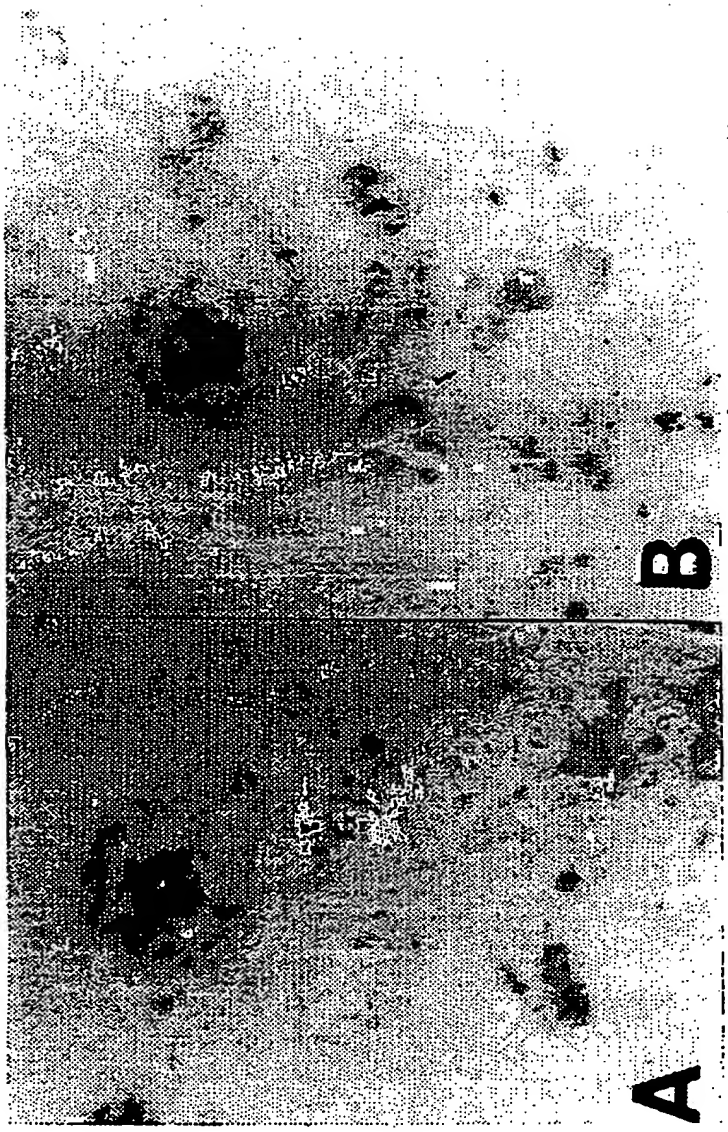

FIG. 9A

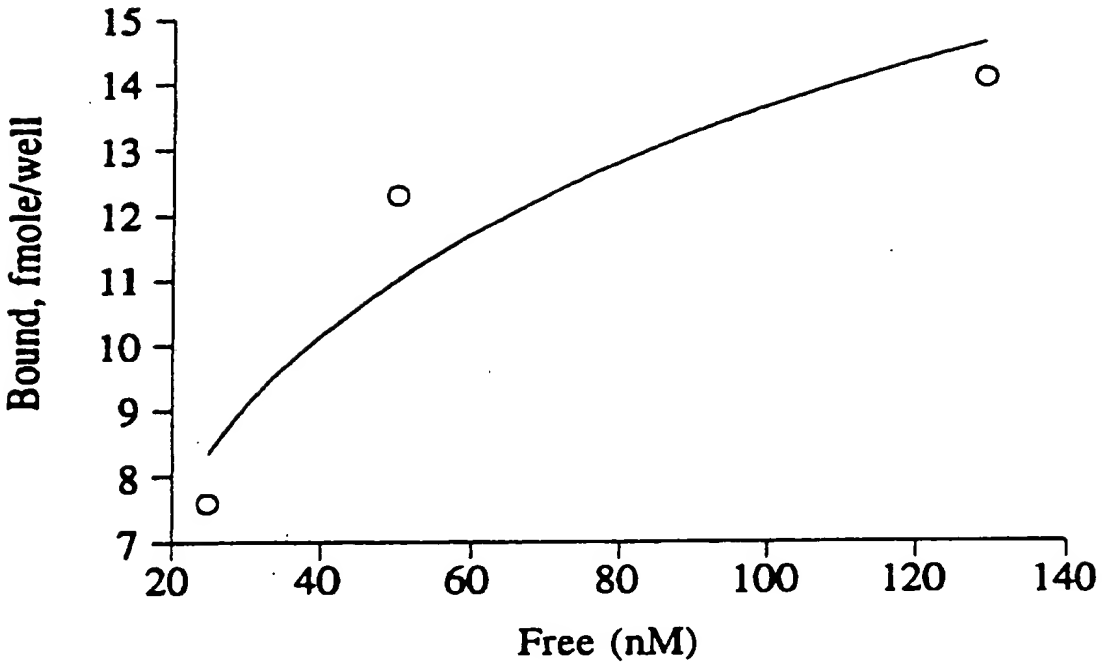
Bound, fmole/well
15
14
13
12
11
10
9
8
7
20
40
60
80
100
120
140
Free (nM)

FIG. 9B

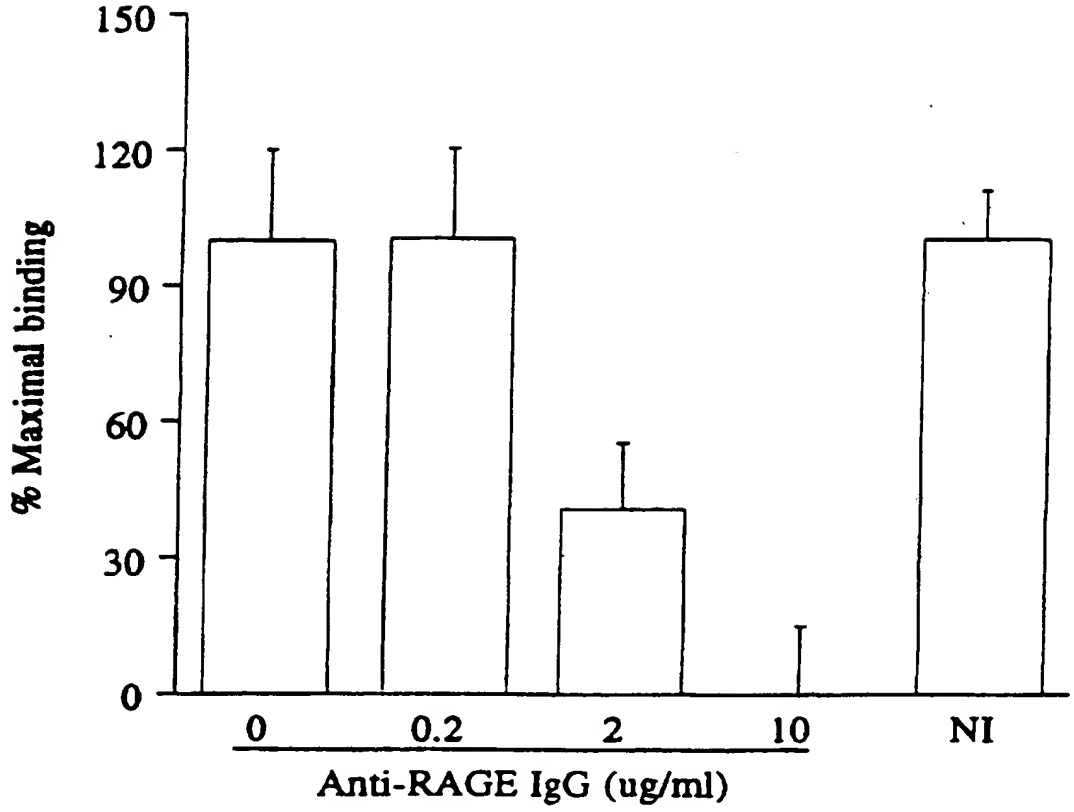
150
120
90
60
30
0
% Maximal binding
0
0.2
2
10
NI
Anti-RAGE IgG (ug/ml)

FIG. 10A

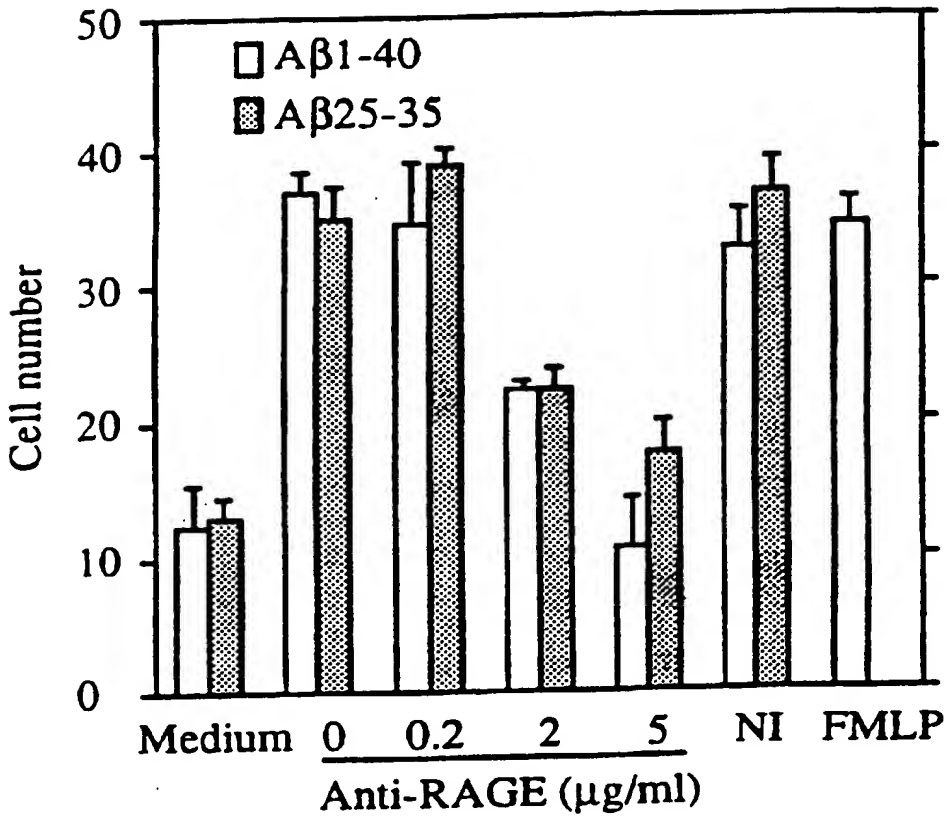
50
40
30
20
10
0
Cell number
Aβ1-40
Aβ25-35
Medium
0
0.2
2
5
NI
FMLP
Anti-RAGE (μg/ml)

FIG. 10B

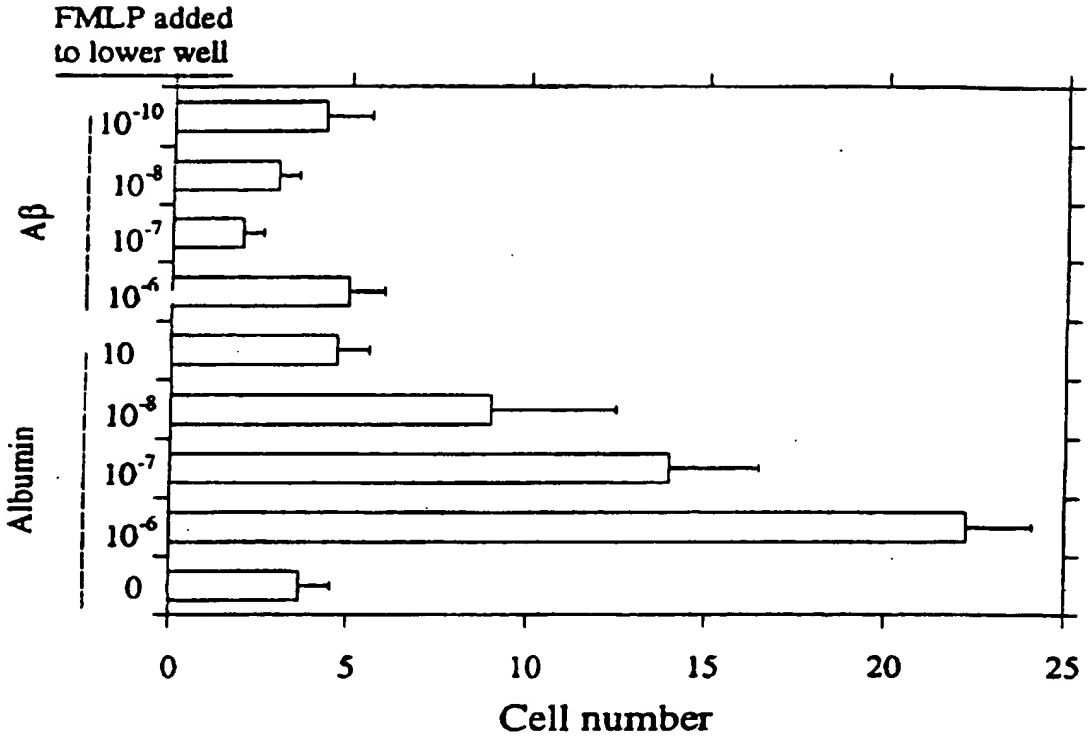

FIG. 10C

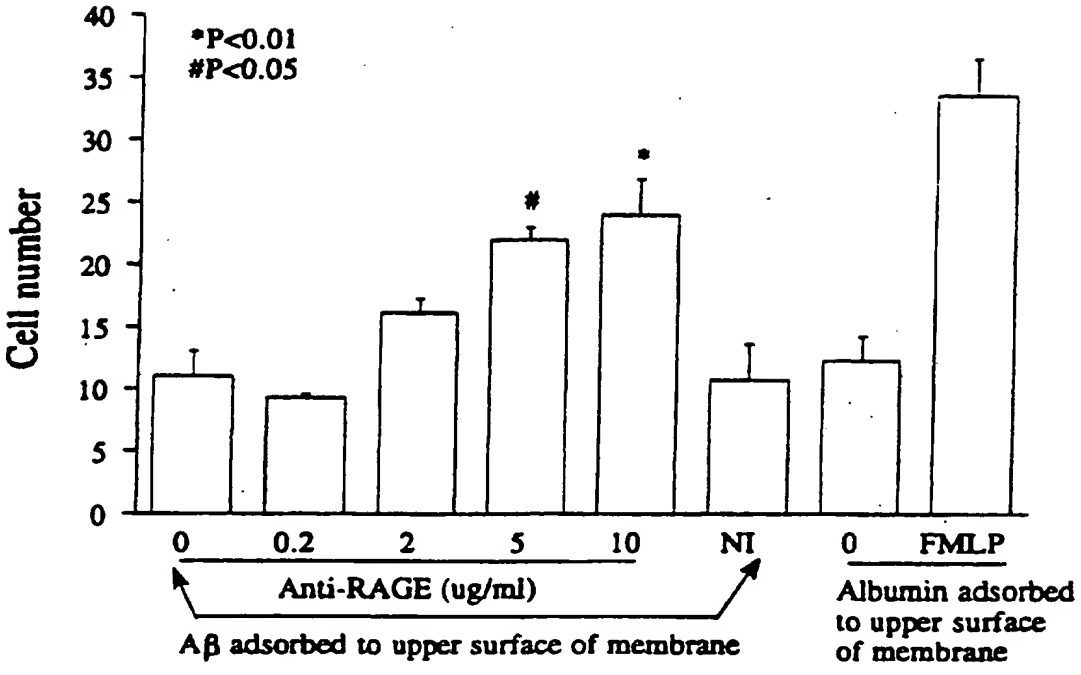
*P<0.01
#P<0.05
Cell number
40
35
30
25
20
15
10
5
0
#
*
0
0.2
2
5
10
NI
0
FMLP
Anti-RAGE (ug/ml)
Aβ adsorbed to upper surface of membrane
Albumin adsorbed to upper surface of membrane

FIG. 11A

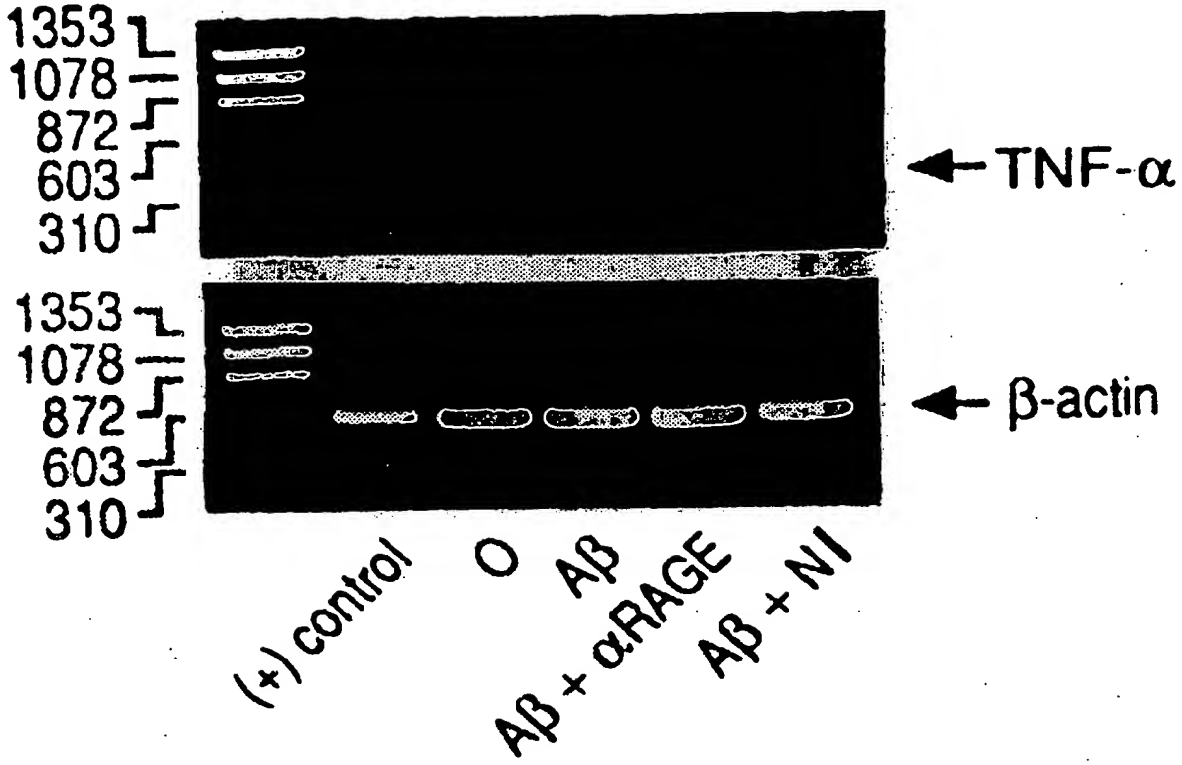

FIG. 11B

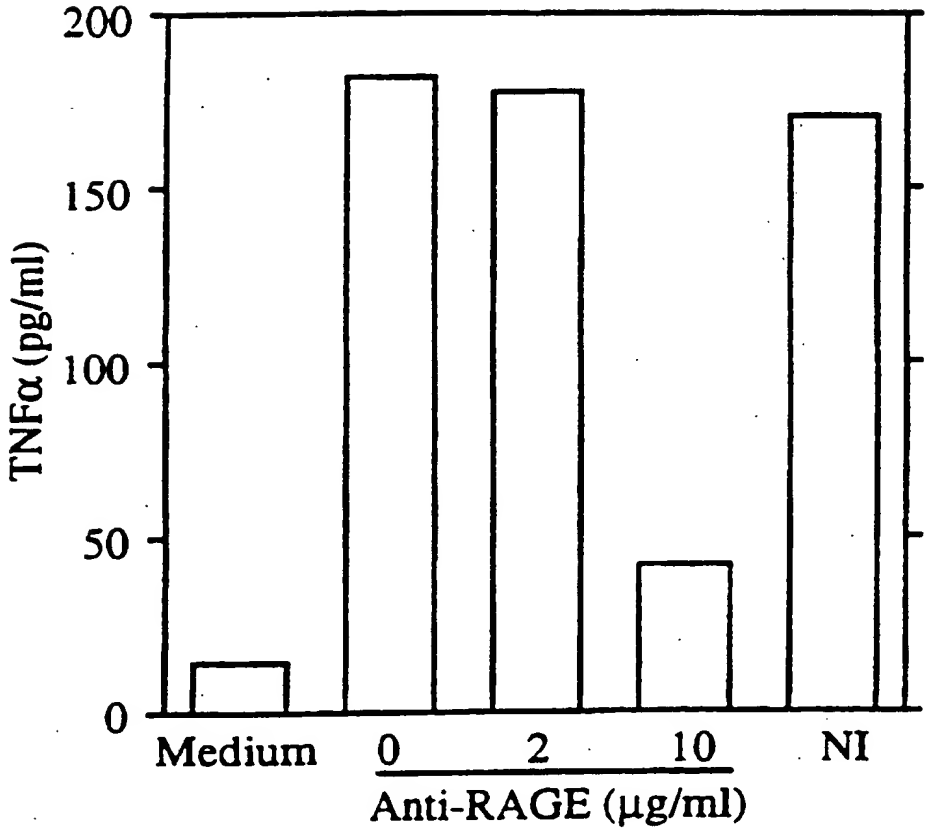

FIG. 11C